APPENDIX A

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	PAT. NO.		Title
1	7,335,633	T	Detecting recurrent vulvovaginal candidiasis or vulvar vestibulitis syndrome and method for treating same
2	7,335,471	T	Polypeptides derived from RNA polymerases and use thereof
3	7,335,363	T	Membrane virus host range mutations and their uses as vaccine substrates
4	7,332,644	T	Non-human animal model of oligodendrocyte developmental disorder
5	7,332,340	T	Process for identifying a novel target for use for the development of therapeutic modalities and drugs effective against tuberculosis
6	7,332,292	T	Constitutively translocating cell line
7	7,332,282	T	Compositions and methods for detecting and treating neurological conditions
8	7,332,174	T	Mutant forms of cholera holotoxin as an adjuvant
9	7,329,795	T	Model animals non-responsive to mycobacterial-origin lipoprotein/lipopeptide
10	7,329,735	T	Fluorescent protein variants and methods for making same
11	7,329,725	T	Phage displayed Trp cage ligands
12	7,329,514	T	Process for producing n-acetylneuraminic acid
13	7,329,486	T	High-throughput assay for virus entry and drug screening
14	7,326,782	T	Metabolic engineering of viomycin biosynthesis
15	7,326,528	T	Method of detecting disease conditions in C. elegans infected with animal virus
16	7,323,624	T	Method for influencing the content of sinapine in transgenic plant cells and in plants
17	7,323,549	T	IL-7 fusion proteins

18 7,323,338 T Plants characterized by an increased content of methionine and related metabolites, methods of generating same and uses thereof
19 7,323,303 T Modified .beta.-lactamases and uses thereof
20 7,320,795 T Rodent hepatitis B virus core proteins as vaccine platforms and methods of use thereof
21 7,319,026 T Amino acid-producing bacteria and a process for preparing L-amino acids
22 7,319,022 T Amplification methods
23 7,319,009 T Methods and compositions for identifying receptor effectors
24 7,317,140 T Sugar and lipid metabolism regulators in plants II
25 7,316,998 T Cerberus/Coco derivatives and uses thereof
26 7,316,903 T Detection of nucleic acid sequence variations using phase Mu transposase
27 7,314,743 T Modified enzymes, methods to produce modified enzymes and use thereof
28 7,314,734 T Muteins of placental growth factor type 1, preparation method and application thereof
29 7,314,713 T Obesity gene and use thereof
30 7,314,631 T Use of recombinant live-attenuated parainfluenza virus (PIV) as a vector to protect against disease caused by PIV and respiratory syncytial virus (RSV)
31 7,312,378 T Nucleic acids encoding heat stable mutants of plant ADP-glucose pyrophosphorylase
32 7,309,598 T gM-negative EHV-mutants
33 7,309,577 T Binding assays that use the T1R1/T1R3 (umami) taste receptor to identify compounds that elicit or modulate umami taste
34 7,309,576 T Method of identifying transmembrane protein-interacting compounds
35 7,307,199 T PCR family genes which confer tolerance to heavy metals
36 7,307,142 T Leptin antagonists
37 7,306,911 T Methods for assessing risk for cardiac dysrythmia in a human subject
38 7,306,901 T Methods and means for assessing HIV envelope inhibitor therapy
39 7,304,205 T DWF12 and mutants thereof
40 7,303,919 T Protein kinase stress-related polypeptides and methods of use in plants
41 7,303,894 T Detection of phenols using engineered bacteria
42 7,301,071 T Method for the production of plant seed with modified fiber content and modified seed coat
43 7,301,068 T Nonhuman model animal of Th2-mediated hyperimmune response
44 7,301,009 T Isolated (T1R1/T1R3) umami taste receptors that respond to umami taste stimuli
45 7,300,781 T Site-directed mutagenesis of Escherichia coli phytase
46 7,300,777 T Feedback-resistant pyruvate carboxylase gene from corynebacterium
47 7,300,762 T Fluorescent proteins and methods of using same
48 7,297,837 T Method for generating hypermutable organisms
49 7,297,772 T Isolated (T1R2/T1R3) sweet taste receptors that respond to sweet taste stimuli
50 7,297,543 T Cell lines that stably or transiently express a functional sweet (T1R2/T1R3) taste receptor

United States Patent **7,335,363**
Hernandez , et al. **February 26, 2008**

Membrane virus host range mutations and their uses as vaccine substrates

Abstract

The present invention is directed to genetically engineered, membrane-enveloped viruses with deletion mutations in the protein transmembrane domains. Also provided are viral vaccines based on the engineered viruses, methods of producing and using such vaccines.

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Related U.S. Patent Documents

Application Number	Filing Date	Patent Number	Issue Date
09952782	Sep., 2001	7128915	
09447103	Nov., 1999	6589533	
09157270	Sep., 1998	6306401	

Current U.S. Class: **424/199.1** ; 424/218.1
Current International Class: A61K 39/12 (20060101)
Field of Search: 424/218.1,199.1

References Cited [Referenced By]

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5843723	December 1998	Dubensky et al.
6306401	October 2001	Brown et al.
6589533	July 2003	Brown et al.
2002/0106379	August 2002	Hernandez et al.

Foreign Patent Documents

WO 99/13818	Mar., 1999	WO
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Government Interests

FEDERAL FUNDING LEGEND

This invention was produced in part using funds obtained through a grant from the National Institutes of Health (AI 42775). Consequently, the federal government has certain rights in this invention.

Parent Case Text

CROSS-REFERENCE TO RELATED APPLICATION

This patent application is a continuation-in-part of U.S. application Ser. No. 09/952,782, filed Sep. 12, 2001, now U.S. Pat. No. 7,128,915, which is a continuation-in-part of U.S. application Ser. No. 09/447,103, filed Nov. 22, 1999 now U.S. Pat. No. 6,589,533, which is a continuation-in-part of U.S. application Ser. No. 09/157,270 filed Sep. 18, 1998, now U.S. Pat. No. 6,306,401.

Claims

What is claimed is:

1. A method of replicating a genetically engineered Arbovirus comprising the steps: a) obtaining a genetically engineered Arbovirus comprising a transmembrane glycoprotein with a deletion of one or more amino acids in a transmembrane domain wherein said engineered Arbovirus has an ability to infect mammalian cells but a reduced ability to replicate therein relative to ***wild type*** virus; b) allowing the virus to replicate in insect cells to produce the engineered Arbovirus.

2. The method of claim 1, wherein the insect cells are mosquito cells.

3. The method of claim 2, wherein the mosquito cells are Aedes albopictus cells.

4. The method of claim 1, wherein the mammalian cells are BHK cells.

5. The method of claim 1, wherein the transmembrane glycoprotein has a deletion of nine or more amino acids in a transmembrane domain.

6. The method of claim 1 wherein the genetically engineered Arbovirus is an Alphavirus.

7. The method of claim 6 wherein said transmembrane glycoprotein is glycoprotein E1, or glycoprotein E2.

8. The method of claim 6, wherein the Alphavirus is a Sindbis virus.

9. The method of claim 8, wherein said Sindbis virus is the TM16 virus.

10. The method of claim 8, wherein said Sindbis virus is the .DELTA.K391 virus.

11. The method of claim 8, wherein said Sindbis virus is the TM17 virus.

12. The method of claim 8, wherein said Sindbis virus is the TM14 virus.

13. The method of claim 1, wherein obtaining a genetically engineered Arbovirus comprises: i) removing nucleotide bases from a region of a viral cDNA clone encoding a transmembrane domain of a viral glycoprotein; ii) transcribing RNA from the cDNA clone; and iii) expressing the RNA into an insect cell to produce a genetically engineered Arbovirus.

14. The method of claim 1, wherein the genetically engineered Arbovirus is a Togavirus.

15. The method of claim 1, wherein the genetically engineered Arbovirus is a Flavivirus.

16. The method of claim 1, wherein the genetically engineered Arbovirus is a Bunya virus.

Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to virology and disease control. Specifically, the present invention relates to mutated arthropod vectored viruses and their uses as vaccines.

2. Description of the Related Art

Arthropod vectored viruses (Arboviruses) are viral agents which are transmitted in nature by blood sucking insects. Arboviruses include members of the alpha-, flavi- and bunyaviridae. Over 600 of these viruses are presently known and emerging members of these families are being described annually. Collectively, the arthropod vectored viruses are second only to malaria as a source of insect-transmitted disease and death in man and animals throughout the world (Berge A. O. 1975). Among these viral agents are Eastern, Western, and Venezuelan Equine Encephalitis Viruses, Dengue Fever, Japanese Encephalititis, San Angelo Fever, West Nile Fever and Yellow Fever. Furthermore,

diseases caused by these agents are in resurgence in North America (NIAID Report of the Task Force on Microbiology and Infectious Diseases 1992, NIH Publication No. 92-3320) as a result of the introduction of the mosquito vector Aedes albopictus (Sprenger, and Wuithiranyagool 1985).

By their very nature, Arboviruses must be able to replicate in the tissues of both the invertebrate insect and the mammalian host (Brown, D. T., and L. Condreay, 1986, Bowers et al. 1995). Differences in the genetic and biochemical environment of these two host cell systems provide a basis for the production of host range mutant viruses which can replicate in one host but not the other.

Currently, Dengue Fever and Eastern Equine Encephalitis and other insect borne viruses are in resurgence in the United States. The U.S. Army and other government agencies have been trying to make vaccines against these viruses since the 1960s with little success. Thus, the prior art is deficient in a vaccine against most arthropod vectored viruses and other membrane-coated viruses. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

Viruses which are transmitted in nature by blood sucking insects are a major source of disease in man and domestic animals. Many of these viruses have lipid membrane bilayers with associated integral membrane proteins as part of their three dimensional structure. These viruses are hybrid structures in which the proteins are provided by the genetic information of the virus and the membrane is the product of the host cell in which the virus is grown. Differences in the composition of the membranes of the mammalian and insect host are exploited to produce virus mutants containing deletions in the membrane spanning domains of the virus membrane proteins. Some of the mutants are capable of replicating and assembling normally in the insect host cell but assemble poorly in the mammalian host cell. These host range mutants produce immunity to wild type virus infection when used as a vaccine in mice, and represent a novel strategy for the production of vaccines against arthropod vectored, membrane containing viruses.

In one embodiment of the present invention, there is provided a genetically engineered membrane-enveloped virus comprising a viral transmembrane glycoprotein that is able to span or correctly integrate into the membrane of insect cells but not that of mammalian cells due to deletion of one or more amino acids in the viral transmembrane glycoprotein. The virus is capable of infecting and producing progeny virus in insect cells, and is capable of infecting but not producing progeny virus in mammalian cells. The virus can be an Arthropod vectored virus such as Togaviruses, Flaviviruses, Bunya viruses and all other enveloped viruses which can replicate naturally in both mammalian and insect cells, as well as enveloped viruses which can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or the cell. Representative examples of such engineered viruses are .DELTA.K391, TM17, TM10 and TM16 viruses.

In another embodiment of the present invention, there is provided a method of producing a viral vaccine by introducing the engineered virus disclosed herein into insect cells and allowing the virus to replicate in the insect cells to produce a viral vaccine. Representative examples of the engineered viruses are .DELTA.K391 virus, TM 17 virus and TM16 virus.

In still another embodiment of the present invention, there is provided a method for vaccinating an individual in need of such treatment comprising the step of introducing the viral vaccine of the present invention into the individual to produce viral proteins for immune surveillance and stimulate immune system for antibody production.

In still yet another embodiment of the present invention, there is provided a method of producing a viral vaccine to a disease spread by a wild mosquito population to mammals, comprising the steps of engineering a deletion of one or more amino acids in a viral transmembrane protein to produce an engineered virus similar to TM16, TM17 or delta K391, wherein the transmembrane protein is able to span the membrane envelope in mosquito cells but not in mammalian cells; introducing the engineered virus, into the wild mosquito population; and allowing the engineered virus to replicate in cells of the wild mosquito population to produce a population of mosquitoes which excludes the wild type pathogenic virus and harbors the vaccine strain of the virus so that a mosquito bite delivers the vaccine to the mammal bitten. Presence of the mutated virus renders the mosquito incapable of transmitting other membrane containing viruses (Karpf et al 1997).

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of one of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention.

FIG. 1 shows the configuration of Sindbis virus glycoproteins after integration into the ER. The protein is a multipass protein with 6 membrane spanning domains (numbered 1-6). 1. The signal sequence for initial integration; 2. The first E2 transmembrane domain (TMD); 3. The second E2 TMD; 4. The first 6k TMD; 5. The second 6k TMD; and 6. The E1 TMD. S=point of cleavage by signal peptidase.

FIG. 2 shows the results of radiolabeled Sindbis virus proteins recovered from transfected tissue-cultured cells. BHK-21 cells mock transfected (1), transfected with mutant .DELTA.391 RNA (2), and Aedes albopictus cells transfected with .DELTA.391 RNA (3), were labeled with radioactive amino acids as described in Example 3. At 24 hours post-transfection, proteins were precipitated with virus specific anti-serum as described in Example 4. The figure shows that both BHK-21 cells and Aedes albopictus cells transfected with RNA of the deletion mutant produce the three viral structural proteins E1, E2, and C which are not detected in the mock transfected cells.

FIGS. 3A and 3B are electron micrographs of BHK-21 cells (FIG. 3A) and Aedes albopictus cells (FIG. 3B) transfected with RNA of the Sindbis virus deletion mutant .DELTA.391. Cells were transfected as described in Example 2. BHK-21 cells (FIG. 3A) show clusters of virus core structures in the cell cytoplasm (A) even though these cells produce very low levels of mature virus (Table 1). Aedes albopictus cells (FIG. 3B) also produce clusters of virus cores; however, these cores are found free in the cells' cytoplasm similar to those in BHK-21 cells (A) and are also found associated with cell membranes (B). This latter case is not found in BHK-21 cells, indicating that the glycoproteins E1 and E2, although present, do not function to bind them.

FIG. 4 shows the deleted amino acids in the E2 transmembranal domain. The deleted sequence is shown under the appropriate amino acid, ranging from 1 to 16 deletions. Histidine and Proline sequences beginning at nt 9717 are on the lumenal side of the protein but are used to design the

mutagenic primers.

FIG. 5 shows circulating Sindbis virus antibody determined by ELISA. Mutant viruses from transfected mosquito U4.4 cells were injected into 25 adult CD-1 mice to establish the protective index (Table 4). Injections of live mutants and UV inactivated viruses were repeated into 3 additional mice to determine Ab titers by standard ELISA. The results presented are from a 10.sup.-2 dilution of mouse serum.

FIG. 6 shows circulating neutralizing antibody. Antiserum used in the experiment described in FIG. 5 was also assayed for neutralizing Ab. The neutralizing Ab data presented represent the % of wild type infectious virus inactivated by a 10.sup.-2 dilution of serum from 3 adult CD-1 mice.

FIG. 7 shows the production of infectious virus by Sindbis mutants having deletions in the transmembrane domain. The numerical designation (e.g. TM10) indicates the number of amino acids remaining. Virus production in insect cells (hatched lines) and mammalian cells (solid lines) are compared to wild type virus (Y420 and SVHR).

FIG. 8 shows the thermal stability of transmembrane mutants. Viruses were heated to the temperature indicated and the treated viruses were titered to determine loss of infectivity.

FIG. 9 shows the particle to plaque forming unit ratios for the transmembrane mutants. The lower the number the more infectious is the virus.

FIG. 10 shows polyacrylamide gel electrophoresis of proteins produced by the transmembrane mutants.

DETAILED DESCRIPTION OF THE INVENTION

It will be apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "membrane-bound virus" refers to a virus which contains a lipid membrane bilayer as part of its protective exterior coat.

As used herein the term "viral envelope" refers to the lipid membrane component of the membrane containing virus and its associated proteins.

As used herein, the terms "arthropod vectored virus" or "Arbovirus" refer to viral agents which replicate and produce progeny virus in arthropod (insect) or mammalian cells. This includes Togaviruses, Flaviviruses and Bunyaviruses.

As used herein, the term "Togavirus" refers to a general classification of membrane containing viruses which include the Alphaviruses.

As used herein, the term "membrane bilayer" refers to a structure consisting of opposed amphiphatic phospholipids. The bilayer is organized in cross section from polar head groups to non-polar carbon chains to nonpolar carbon chains to polar head groups.

As used herein, the term "glycoprotein transmembrane region" refers to the amino acid sequence of the

region of a membrane-integrated protein which spans the membrane bilayer.

As used herein, the term "viral vaccine" refers to a strain of virus or virus mutant which has the antigenic properties of the virus but cannot produce disease.

As used herein the term "immune surveillance" refers to a process by which blood lymphocytes survey the cells and tissues of a mammal to determine the presence of foreign (virus) proteins and stimulates the production of lymphocytes capable of targeting cells producing the foreign protein for destruction. This process also leads to the production of circulating antibodies against the foreign protein.

As used herein, the term "infectious virus particles" refers to viruses which are capable of entering a cell and producing virus protein, whether or not they are capable of producing progeny virus.

As used herein, the term "non-infectious virus particles" refers to viruses which are not capable of infecting or entering a cell.

As used herein, the term "vertebrate cells" refers to any mammalian cell.

As used herein, the term "invertebrate cells" refers to any insect cell.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D. N. Glover ed. 1985); "Oligonucleotide Synthesis" (M. J. Gait ed. 1984); "Nucleic Acid Hybridization" (B. D. Hames & S. J. Higgins eds. (1985)); "Transcription and Translation" (B. D. Hames & S. J. Higgins eds. (1984)); "Animal Cell Culture" (R. I. Freshney, ed. (1986)); "Immobilized Cells And Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

The vaccines of the present invention are based on deletion mutations in the transmembrane domains of membrane glycoproteins of membrane-enveloped viruses. Many membrane-coated viruses have membrane glycoproteins on their surface which are responsible for identifying and infecting target cells (Schlesinger, S. and M. J. Schlesinger, 1990). These membrane glycoproteins have hydrophobic membrane-spanning domains which anchor the proteins in the membrane bilayer (Rice et al 1982).

The membrane-spanning domains of these transmembrane proteins must be long enough to reach from one side of the bilayer to the other in order to hold or anchor the proteins in the membrane. Experiments have shown that if the domains are shortened by the deletion of amino acids within the domain, the proteins do not appropriately associate with the membrane and fall out (Adams and Rose. 1985).

Unlike mammalian cell membranes, the membranes of insect cells contain no cholesterol (Clayton 1964, Mitsuhashi et al 1983). Because insects have no cholesterol in their membranes, the insect-generated viral membrane will be thinner in cross section than the viral membranes generated from mammals. Consequently, the membrane-spanning domains of proteins integrated into insect membranes do not need to be as long as those integrated into the membranes of mammals. It is possible, therefore, to produce deletions in engineered viruses which remove amino acids from the transmembrane domain of the viral glycoprotein. This results in a glycoprotein which can integrate normally into the membrane of a virus replicating in an insect cell, but not into the membrane of a

virus replicating in a mammal. Thus, the mutated virus can replicate and be produced in insect cells as well as the parent wild-type virus. On the other hand, the mutant virus can infect mammalian cells and produce viral proteins; however, since the mutated virus glycoprotein cannot span and be anchored in the mammalian membrane, progeny virus cannot be produced in mammalian cells. An advantage to the approach of the present invention is that the mutants are engineered as deletion mutants, hence there is absolutely no chance for reversion to wild-type phenotype, a common problem with virus vaccines.

The protocol described by the present invention works for any virus which replicates in insects and mammals and has integral membrane proteins as part of its structure, namely, Togaviruses, Flaviviruses and Bunya viruses and all other enveloped viruses which can replicate naturally in both mammalian and insect cells, as well as enveloped viruses which can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or the cell.

Vaccines are made against any membrane-containing virus by removing amino acids from the membrane-spanning domain of a protein in the viral envelope. This is done by removing bases from a cDNA clone of the virus as described below. RNA transcribed from the altered clone is transfected into insect cells. The viruses produced are amplified by repeated growth in insect cells until large quantities of mutant viruses are obtained. These viruses are tested for its ability to infect and produce progeny in mammalian cells. Viruses which do not produce progeny in mammalian cells are tested for ability to produce immunity in laboratory animals. Those viruses which do produce immunity are candidates for production of human and animal vaccines by procedures known in the art.

Using the prototype of the Alphaviridea, Sindbis virus, the different compositions of insect and mammalian membranes are exploited to produce mutants which assemble efficiently in insect cells but assemble poorly in mammalian cells. The envelope glycoproteins of Sindbis virus are integrated into the membranes of the endoplasmic reticulum as a multi pass protein with 6 membrane spanning domains. There are, therefore, 6 potential targets for the production of deletion mutations which will prevent the correct integration of a transmembrane domain (TMD) (See FIG. 1). Some of these targets are less satisfactory for deletion mutagensis because they have functions other than simply anchoring the protein in the membrane bilayer. For example, transmembrane domain #1 (FIG. 1) is the signal sequence which is recognized by the Signal Recognition Particle and directs protein synthesis to the membranes of the endoplasmic reticulum. Truncating this domain would likely disturb targeting in both mammalian and insect cells. TMD #3 will become a cytoplasmic domain upon protein maturation and contains specific sequences that recognize and bind capsid protein. It has been shown that this interaction is very specific in nature and requires the sequence that is in the transmembrane domain (Liu et al., 1996; Lopez et al., 1994). TMD #3, therefore, like TMD #1 has a functional as well as a structural component. A significant deletion in this domain would likely eliminate budding in both cell systems. This leaves four transmembrane domains as targets for the production of deletions which will effect membrane integration (FIG. 1, TMD #2, #4, #5, and #6).

The 6k protein is not a component of mature virus and its function in virus assembly is not clear. In the poly protein the proper integration and orientation of 6k in the endoplasmic reticulum membrane is essential for the correct integration of E1. The transmembrane domains of 6k (TMD #4 and #5) are excellent targets for deletion mutation as failure to integrate one of these domains may cause the poly protein to integrate into the membrane in a wrong configuration or cause the failure to integrate E1. TMD #2 and #6 are the membrane spanning domains of E2 and E1 and are both obvious targets for deletion mutation. Multiple membrane spanning domains in this poly protein suggest that if deletion mutations in a single transmembrane domain do not totally block virus production in mammalian cells,

then deletions in additional membrane spanning domains can further reduce maturation to negligible levels.

The present invention is directed to a genetically engineered membrane-enveloped virus comprising a transmembrane protein which has a deletion of one or more amino acids in the transmembrane region of the protein such that the transmembrane protein is able to span or correctly integrate into the membrane of an infected cell when the engineered virus replicates in insect cells, but is unable to span or integrate into the membrane of an infected cell when the virus replicates in mammalian cells. Preferably, the virus is an Arthropod vectored virus selected from the group consisting of Togaviruses, Flaviviruses, Bunya viruses and all other enveloped viruses which can replicate naturally in both mammalian and insect cells, as well as enveloped viruses which can be made to replicate in mammalian and insect cells by genetic engineering of either the virus or the cell. Representative examples of such engineered viruses are .DELTA.K391, TM17, TM10 and TM16 viruses. Preferably, the insect cells are mosquito cells, such as Aedes albopictus cells, and the mammalian cells are human cells.

In a preferred embodiment, the genetically engineered, membrane-enveloped virus is Sindbis virus, and the transmembrane protein is viral glycoprotein E2. However, a person having ordinary skill in this art could readily predict that similar mutations can be successfully installed in the membrane spanning domains of other virus membrane proteins such as E1.

In another preferred embodiment, the genetically engineered membrane-enveloped virus is selected from the group consisting of HSV, HIV, rabies virus, Hepatitis, and Respiratory Syncycial virus, and the transmembrane protein is selected from the group consisting of glycoprotein E1, glycoprotein E2, and G protein.

In still another preferred embodiment, the genetically engineered membrane-enveloped virus are RNA tumor viruses, and the transmembrane protein is Env.

The present invention is also drawn to a method of producing a viral vaccine from the genetically engineered membrane-enveloped virus disclosed herein for vaccination of mammals, comprising the steps of introducing the engineered virus into insect cells and allowing the virus to replicate in the insect cells to produce a viral vaccine. Representative examples of the engineered viruses are .DELTA.K391 virus, TM17 virus and TM16 virus.

In addition, the present invention provides a method of vaccinating an individual in need of such treatment, comprising the steps of introducing the viral vaccine of the present invention into the individual and allowing the vaccine to produce viral proteins for immune surveillance and stimulate immune system for antibody production in the individual.

Furthermore, the present invention provides a method of producing a viral vaccine to a disease spread by a wild mosquito population to a mammal, comprising the steps of genetically engineering a deletion of one or more amino acids in a viral transmembrane protein to produce an engineered virus, wherein the transmembrane protein is able to span the membrane envelope when the virus replicates in mosquito cells, but is unable to span the membrane envelope when the virus replicates in mammalian cells, and wherein the virus remains capable of replicating in mosquito cells; introducing the engineered virus into a wild mosquito population; and allowing the virus to replicate in cells of the wild mosquito population to produce a population of mosquitoes which excludes the wild type pathogenic virus and harbors the vaccine strain of the virus such that the mosquito bite delivers the

vaccine to a mammal bitten.

It is contemplated that pharmaceutical compositions may be prepared using the novel mutated viruses of the present invention. In such a case, the pharmaceutical composition comprises the novel virus of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art readily would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of this viral vaccination compound. When used in vivo for therapy, the vaccine of the present invention is administered to the patient or an animal in therapeutically effective amounts, i.e., amounts that immunize the individual being treated from the disease associated with the particular virus. It will normally be administered parenterally, preferably intravenously or subcutaineusly, but other routes of administration will be used as appropriate. The amount of vaccine administered will typically be in the range of about 10.sup.3 to about 10.sup.6 pfu/kg of patient weight. The schedule will be continued to optimize effectiveness while balancing negative effects of treatment. See Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Pa.; and Goodman and Gilman's: The Pharmacological Basis of Therapeutics 8th Ed (1990) Pergamon Press; which are incorporated herein by reference. For parenteral administration, the vaccine will be most typically formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

A Single Amino Acid Deletion Mutant, K391

Using the full length clone of the Alpha virus Sindbis described previously (Liu et al 1996, Rice et al., 1987), a deletion removing 3 bases encoding a lysine at position 391 in the amino acid sequence of the virus glycoprotein E2 has been constructed. This lysine is part of the putative membrane-spanning domain of this protein (Rice et al 1982).

Site-directed mutagenesis was used to generate a deletion mutant (Lys391) in Toto 1101, a plasmid containing the full-length Sindbis cDNA and an SP6 promoter that can be used to transcribe infectious RNA from the clone in vitro (Rice et al., 1987; Liu and Brown, 1993a). Using the megaprimer method of PCR mutagenesis (Sarkar and Sommer, 1990) described previously (Liu and Brown, 1993a), three nucleotides (nucleotides 9801, 9802, 9803) were removed in the cDNA clone of Toto 1101, resulting in the removal of the codon AAA (K391).

A 30 base oligonucleotide of the sequence, 5'CTCACGGCGCGCACAGGCACATAACACTGC3' (SEQ ID No.: 1) was used as the mutagenesis primer. This primer, along with the "forward primer" 5'CCATCAAGCAGTGCGTCG3' (SEQ ID No.: 2; 18mer), generated a 518 base "Megaprimer" (nucleotides (nts) 9295-9813). The second PCR reaction consisted of 0.5 .mu.g of megaprimer, 100 .mu.g Toto 1101 template and 0.5 .mu.g of the "reverse primer" 5'GGCAGTGTGCACCTTAATCGCCTGC 3' (SEQ ID No.: 3). All PCR reactions employed 30 cycles at 95 degrees for 1 min., 64 degrees for 1 min., 72 degrees for 1 min. and a final incubation at 72 degrees for 8 min. The resulting PCR product (1149 nts) was cleaved with BCL I and SPL and inserted into the corresponding site in Toto 1101, creating the deletion mutant K391. After the deletion

was confirmed by dideoxynucleotide sequencing through the entire subcloned region using Sequenase.TM. (U.S. Biochemical, Cleveland, Ohio), infectious RNA was transcribed in vitro using SP6 polymerase and was introduced into BHK-21 cells.

EXAMPLE 2

In Vitro Transcription and RNA Transfection of K391

Plasmid DNA containing the full-length cDNA copy of Sindbis virus K391 or wild type RNA was linearized with XhoI and transcribed in vitro with SP6 RNA polymerase as described previously (Rice et. al., 1987). 1 .mu.g of Xho I linearized K391 cDNA or wild type Sindbis virus cDNA was transcribed in buffer consisting of 80 mM Hepes, pH 7.5, 12 mM MgCl, 10 mM DTT, 2 mM spermidine and 100 .mu.gm BSA with 3 mM each ATP, UTP, CTP, 1.5 mM GTP and 4.5 mM m.sup.7 GpppG, 20 units SP6 RNA polymerase and 20 units RNase inhibitor in a 20 .mu.l reaction volume. After incubation at 37.degree. C. for 2 hours, RNA production was assayed by running 2 .mu.l of the RNA product on a 1% agarose gel.

Baby Hamster Kidney (BHK21) cells and Aedes albopictus (mosquito) cells were transfected with RNA derived from the mutant or wild type clone. Mosquito cell transfections were carried out using 5.times.10.sup.6 cells resuspended in RNase free electroporation buffer consisting of 20 mM Hepes pH 7.05, 137 mM NaCl, 0.7 mM Na.sub.2HPO.sub.4 and 6 mM dextran. Washed cells were resuspended in diethyl pyrocarbonate (DEPC) treated water to a concentration of 5.times.10.sup.7 cells/ml. RNA transcripts in 20 .mu.l were added to 400 .mu.l washed cells and transferred to a 0.2 cm gap length cuvette. Optimal electroporation parameters for these cells was found to be 2 KV 25 .mu.F, 8 resistance. Transfected cells were incubated at 37.degree. C. until cytopathic effect was observed (about 24 hours).

After 24 hours of incubation, the media was collected from both infected cell lines as well as non-RNA transfected controls. The media from each cell line was tested for the presence of infectious virus by plaque assay (as described by Renz and Brown 1976) on mosquito and BHK-21 cell monolayers (Table 1).

TABLE-US-00001 TABLE 1 Infectious virus produced by transfection of BHK21 or Aedes albopictus (AA) cells with Sindbis virus wild type (wt) or mutant K391 BHK BHK AA Mock.sup.a BHK with AA Mock AA with Cell line Trans- with K391 Trans- with K391 transfected fected wt RNA RNA fected wt RNA RNA Media no virus 1.5 .times. 3.0 .times. no virus 5.0 .times. 1.0 .times. titered detected 10.sup.9 10.sup.3 detected 10.sup.8 10.sup.8 on BHK virus/ml virus/ml Media no virus 8 .times. 8.0 .times. no virus 1.0 .times. 2.0 .times. titered detected 10.sup.7 10.sup.4 detected 10.sup.9 10.sup.9 on AA virus/ml virus/ml virus/ml .sup.aMock indicates that transfection protocol was carried out without RNA

As shown in Table 1, the mutant K391 produces significant amounts of infectious virus particles only when replicating in the insect cell. BHK cells transfected with K391 produced very low levels of virus, 4 to 5 orders of magnitude lower than the amount produced in insect cells.

EXAMPLE 3

Metabolic Radioactive Labeling of Viral Proteins

Subconfluent monolayers of BHK21 cells in 25 cm.sup.2 flasks were transfected with wild type or K391 mutant RNA as described above. Monolayers were starved for 30 min in methionine- and cysteine-free medium (MEM-E) containing 1% FCS, 2 mM glutamine and 5% TPB (starvation medium). At 16 hours post-transfection, cells were pulse-labeled with starvation medium containing 50 .mu.Ci/ml [.sup.35S] Met/Cys protein labeling mix for 20 minutes. Labeling was terminated by washing the monolayers with PBS containing 75 .mu.g/ml cycloheximide. Monolayers were chased for 45 minutes in medium containing 10 times the normal concentration of methionine and cysteine and 75 .mu.g/ml cycloheximide.

EXAMPLE 4

Immunoprecipitation and Polyacrylamide Gel Electrophoresis

Radiolabeled viral proteins were immunoprecipitated with antisera as described (Knipfer and Brown, 1989). [.sup.35S] Met/Cys labeled cells were washed twice in cold PBS and lysed in lysis buffer: 0.5% NP-40, 0.02 M Tris HCl pH 7.4, 0.05 M NaCl, 0.2 mM PMSF, 0.2 mM TPCK and 0.02 mM TLCK. The nuclei were pelleted by centrifugation and discarded. The supernatant was pre-absorbed with 100 .mu.l of protein A/Sepharose beads (Sigma) suspended in lysis buffer for 1 hr, and the beads were pelleted. The pre-absorbed supernatant was treated with 200 .mu.l of protein A/Sepharose beads coupled to rabbit anti-SVHR serum or E2 tail monospecific polyclonal serum and agitated overnight at 4.degree. C. The immunoprecipitated bead-antibody-protein complexes were washed three times with lysis buffer and then solubilized in SDS-PAGE sample buffer consisting of 12% glycerol, 4% SDS, 50 mM Tris pH 6.8, 5% mercaptoethanol and 0.02% bromphenol blue. The samples were heated for 3 min at 95.degree. C. and the beads were removed from the sample by centrifugation. Gel electrophoresis was carried out on a 10.8% SDS-PAGE or 16% Tricine gel as described previously (Liu and Brown, 1993 a,b). Fluorography was performed as described (Bonner and Laskey, 1974) and dried gels were exposed to Kodak XAR-5 film (see FIG. 2).

EXAMPLE 5

Transmission Electron Microscopy

BHK-21 cell monolayers infected with K391 produced from transfected mosquito cells or transfected with K391 RNA were lifted from flasks by trypsin treatment at desired time points, and the cells were pelleted by low speed centrifugation. Cell pellets were washed twice in PBS and fixed in 4% glutaraldehyde at 4.degree. C. overnight. The cells were then washed three times with 0.2 M cacodylate buffer (pH 7.2), post-fixed with 2% osmium tetroxide for 1 hour at room temperature, and washed three times in cacodylate buffer. The cells were stained en bloc for 1 hr at room temperature with 0.5% uranyl acetate. After three washes, cell pellets were embedded in 1% agarose and dehydrated through a graded ethanol/acetone series. Final embedding was in Mollenhauer's (1964) Epon-Araldite epoxy mixture #1 at 70.degree. C. for two days. Ultrathin sections were cut on a Sorvall MT5000 microtome and collected on 150 mesh copper grids. Sections were stained with 1% uranyl acetate and/or lead citrate and were photographed in a Jeol 100CX transmission electron microscope (see FIG. 3).

Although BHK cells infected with K391 virus or transfected with K391 RNA produce no virus detectable by the plaque assay, it was shown by PAGE that they do produce all virus structural proteins (FIG. 2). Further, it was shown by electron microscopy that they assemble the intracellular (non infectious) virus cores (FIG. 3).

Delta K391 produces very high titers of mutant Sindbis virus particles when allowed to replicate in mosquito cells. The exposed regions of the proteins (ecto domains) are wild type in sequence. These wild type proteins allow the virus to enter mammalian cells and produce virus proteins (see FIG. 2) but new virus is not assembled as shown by electron microscopy in FIG. 3.

Delta K391 is a vaccine strain. It is produced in very high concentration in cultured insect cells. When the virus is injected into a mammalian host, the virus circulates and infects cells in the mammalian host. These infected cells produce and present virus proteins for immune surveillance. However, the infection will be limited primarily to those cells infected initially by the innoculum because of the truncation in the membrane domain of the viral glycoprotein. Because the vaccine strain is the result of a deletion mutation, reversion to wild type pathogenic phenotype is not possible.

Furthermore, an engineered deletion mutant may be introduced into the wild mosquito population. It has been shown that these viruses are spread from the female parent to the progeny by a process of transovariol transmission (Leakey 1984). When these mosquitoes bite a vertebrate they will provide an immunizing dose (10.sup.6 infectious particles) of the vaccine strain (for example, Delta K391). Karpf et al (1997) showed that infection of insect cells by one Alpha virus prevents the cells from being infected by another, even distantly-related alpha virus for an indefinite amount of time (over two years in cell culture, where the life of a mosquito is 28 days). Thus, the presence of the vaccine strain such as K391 or other deletion mutants described in the present invention will block the spread of other related and pathogenic viruses by these insects.

EXAMPLE 6

Deletion in the E2 Transmembrane Domain

Protocols for testing the requirements placed on the transmembrane domain of E2 (FIG. 1, transmembrane domain #2) is given. This protocol can be easily replicated for any other of the Sindbis membrane spanning domains or the membrane spanning domains of any other virus glycoprotein. The hydrophobic Sindbis PE2 membrane anchor consists of 26 amino acids. As is common with other membrane spanning domains little amino acid homology is conserved among the alphaviruses, although the length of this hydrophobic region is highly conserved (Strauss and Strauss, 1994). The lack of sequence conservation in this domain suggests that it is the hydrophobic properties of the domain and not its sequence which is critical for integration.

The transmembrane domain of E2 begins at amino acid 365 of the PE2 sequence. This hydrophobic region consists of the sequence: VYTILAVASATVAMMIGVTVAVLCAC (SEQ ID No.: 4). Adams and Rose (1985) demonstrated that a minimum of 14 amino acids in the transmembrane domain of the VSV G protein were necessary for proper anchoring in mammalian cells. Therefore, mutagenic primers have been designed which create a nested set of deletions in the E2 transmembrane domain. Beginning with a deletion of 16 amino acids (which leaves 10 amino acids in the hydrophobic region), a set of deletions were constructed which delete from as many as 16 amino acids, to as few as 1 amino acid from the membrane anchor (FIG. 4).

Deletions were constructed using PCR megaprimer mutagenesis to generate deleted fragments containing unique BclI and SplI sites. All resulting constructs were installed into the wild-type Sindbis cDNA construct Toto Y420 to generate the mutant plasmids. After linearization with XhoI and transcription using SP6 polymerase, transcripts were transfected into BHK or Aedes albopictus cells

by electroporation as described above. Production of infectious virus from these transfections were titered on both BHK and C710 mosquito cells to determine the host range of these constructs. Table 2 shows the deleted sequences and the primer sequences used in their construction.

For each construct the same primer pair is used to generate the entire BclI to SplI region. The forward primer E1Bcl21 is comprised of the sequence from nucleotide 9306-9327 and reads from 5'-3' GCGTCGCCTATAAGAGCGACC (SEQ ID No.: 5). The reverse primer Splext is comprised of the sequence from nucleotide 10420-10444 which is the complementary sequence reading from 5'-3 CAGTGTGCACCTFAATCGCCTGC (SEQ ID No.: 6).

The virus produced by transfection of insect cells is tested for its ability to produce plaques in BHK and C7-10 mosquito cells as for the mutant E2 .DELTA.K391. Those mutants which do not produce plaques in BHK cells are tested for their ability to infect BHK cell relative to wild type virus by immunofluorescence assay of infected monolayers. This later assay is compared to the total protein in purified preparations of the mutant and wild type virus to establish the relative infectivity of each mutant population. The goal is to truncate the transmembrane domain as much as possible and still obtain reasonable amounts of virus in C7-10 mosquito cell monolayers which can infect but not produce mature virus in BHK cells. Additional transmembrane domains (up to four domains) can be truncated in circumstances where truncation of a single transmembrane domain reduces but does not eliminate virus growth in BHK cells.

The length of the transmembrane (TM) domain of E2 was systematically reduced from 26 amino acids to 10, 12, 14, 16, 17 and 18 amino acids, and the effects of these truncations on the ability of these viruses to replicate in cells of the vertebrate (BHK-21, hamster cells) and invertebrate (Aedes albopictus, mosquito cells) hosts were examined.

Table 3 presents results typical of several of such experiments. The data reveal that reducing the transmembrane domain from 26 to 10 amino acids or 12 amino acids results in viruses incapable of efficient assembly in either host. Increasing the length of the transmembrane domain to 14 amino acids results in viruses that grow poorly in mammalian cells but somewhat better in insect cells. Increasing transmembrane length to 16 or 17 amino acids restores wild type levels of growth in insect cells while growth in mammalian cells remains greatly impaired. Increasing the length of the transmembrane domain to 18 amino acids restores growth in mammalian cells. The reduction in the length of the transmembrane domain of the E2 glycoprotein has resulted in the production of virus mutants in which efficient growth is restricted to insect cells. The accepted terminology for such mutations is "host range mutation".

The data presented above show that large deletions in the transmembrane (TM) domains of the glycoproteins of insect vectored viruses can result in the restriction of virus assembly to insect cells. Mutants which produce low levels of virus (TM 10, 12, 14) are unable to correctly integrate the membrane proteins into the host cell membranes. The less impaired mutants, represented by TM 16 and TM 17, can infect mammalian cells, produce structural proteins, and form nucleocapsid structures containing the viral RNA. However, these mutants are defective in steps in virus assembly.

TABLE-US-00002 TABLE 2 Listing of the deletions in Sindbis E2 and the primers used Primer-Designated by No. of Nucleo- Transmembranal tides Oligonucleotide Sequence of Amino Acids Deleted Mutagenic Primer (Negative Strand) E2 TM10 9734-9782 ACATAACACTGCGATGGTGTACAC (SEQ ID No.: 7) E2 TM12 9740-9782 ACATAACACTGCGGCTAAGATGG (SEQ ID No.: 8) E2 TM14 9746-9782

ACATAACACTGCTGCGACGGCT (SEQ ID No.: 9) E2 TM16 9743-9773
GCAACAGTTACGACGGCTAAG (SEQ ID No.: 10) E2 TM17 9743-9770
ACAGTTACGCCGACGGCTAAG (SEQ ID No.: 11) E2 TM18 9743-9767
GTTACGCCAATGACGGCTAAG (SEQ ID No.: 12) E2 TM19 9743-9764
CGCCAATCATGACGGCTAAGA (SEQ ID No.: 13) E2 TM20 9755-9773
GCAACAGTTACGGTAGCTGA (SEQ ID No.: 14) E2 TM21 9755-9770
AGTTACGCCGGTAGCTGA (SEQ ID No.: 15) E2 TM22 9761-9773
TGCAACAGTTACCGCCACGGT (SEQ ID No.: 16) E2 TM23 9761-9770
ACAGTTACGCCCGCCACGGT (SEQ ID No.: 17) E2 TM24 9761-9767
GTTACGCCAATCGCCACGGT (SEQ ID No.: 18) E2 TM25 9761-9764
ACGCCAATCATCGCCACGGT (SEQ ID No.: 19)

TABLE-US-00003 TABLE 3 Growth of Sindbis virus TM deletion mutants in insect and vertebrate cells Growth in insect cells Growth in mammalian cells Mutant.degree..sup..phi. (pfu/ml).sup..dagger. (pfu/ml).sup..dagger-dbl. Wild type 5 .times. 10.sup.9 5 .times. 10.sup.9 TM10 2 .times. 10.sup.3 3 .times. 10.sup.4 TM12 5 .times. 10.sup.3 6 .times. 10.sup.2 TM14 6 .times. 10.sup.7 4 .times. 10.sup.2 TM16 2 .times. 10.sup.9 7 .times. 10.sup.4 TM17 3 .times. 10.sup.9 1 .times. 10.sup.5 TM18 1 .times. 10.sup.8 6 .times. 10.sup.8

Mutants were constructed using the Stratagene Quick change.RTM. mutagenesis protocol using a cDNA template containing the virus structural genes. Desired mutations were subcloned into the full length virus cDNA vector containing an SP6 promoter for the transcription of full length infectious viral RNA. Mutant transcripts were transfected into .sup..dagger. mosquito cells or .sup..dagger-dbl. mammalian BHK cells and incubated for the appropriate time and temperature before harvesting the virus-containing media. Virus yields from both cell types were assayed by titration on monolayers of BHK cells.

EXAMPLE 7

Uses of Deletion Mutants as Vaccine

Mutations which restrict the assembly of virions only to insect cells suggest that viruses produced from these cells may be used to infect an animal which could only produce low numbers of progeny viruses. Such a phenotype could result in the production of protective immunity in that animal without pathological consequences. Mutants TM 16 and 17 were selected for further study to determine their potential for producing protective immunity. The results of these experiments are presented in Table 4.

TABLE-US-00004 TABLE 4 Protection Of Adult Mice From Sindbis Virus By Vaccination With TM Mutations Mor- Mor- bitity tality POST POST MOR- MOR- CHAL- CHAL- CHAL- VIRUS DOSE TALITY BIDITY LENGE LANGE LENGE Mock 10.sup.6 0% 0% SAAR86 92% 36% (buffer) 1000 pfu i.c. TM16 10.sup.6 0% 0% SAAR86 68% 48% 1000 pfu i.c. TM16 NA 0% 0% SAAR86 92% 36% UV 1000 pfu i.c. TM17 10.sup.6 0% 0% SAAR86 0 0 1000 pfu i.c. TM17 NA 0% 0% SAAR86 84% 36% UV 1000 pfu i.c.

Twenty five 21 days old CD-1 mice were used in each study. Mutant viruses from transfected Aedes albopictus U4.4 cells was injected into the mice subcutaneously at the dose indicated. Fourteen days after the initial injection the mice were challenged with the SAAR 86 strain of Sindbis virus as indicated.

TM 16 was a poor vaccine compared to TM 17 although both mutations showed identical phenotypes in the tissue culture cell system described in Table 0.3. It is clear that the protection achieved by injection with TM 17 was not the simple result of exposure to virus protein, as the UV treated virus did not protect. To further elucidate the mouse response to these two mutants, serum of vaccinated mice was tested for the presence of circulating antibody by standard ELISA assay. The results of this experiment is shown in FIG. 5. Mutants TM16 and TM 17 appeared to produce similar levels of circulating antibody as would be expected from an inoculation with the same quantity of virus. This result suggested that antibodies capable of binding to denatured virus, as well as infectious virus, were induced in the mice at roughly equivalent levels by both mutants.

The results presented in FIG. 5 suggested that the immune response to TM 17 was different from the response to TM16. The serum of the vaccinated mice was therefore examined for the presence of neutralizing antibody to Sindbis virus. The results are shown in FIG. 6. By contrast with mutant TM16, mutant TM 17 induced significantly more neutralizing antibody. This likely explains its superior performance as a vaccine.

The transmembrane domains of the glycoproteins of Alpha, Flavi and Bunya viruses which have been sequenced reveal that they have the common property of being hydrophobic sequences that are predicted to form alpha helices in membrane bilayers. It is predicted that truncation of the transmembrane domain described above for an alphavirus will produce a similar pattern of host restriction in any one of these viruses. Thus, the protocol described above has the potential of producing live vaccines against any one of these agents. Because the mutations are large deletions, there is little prospect of spontaneous reversion to wild type virus. Indeed, in the time these mutants were examined in the laboratory, no such revertants have been detected.

The observation that TM16 and 17 have such different properties in terms of their ability to produce protection as a vaccine while having similar growth characteristics in cell culture is most interesting. The data showing that TM 17 produces a higher level of neutralizing antibody suggests that TM 17 may be structurally more identical to wild type virus than TM 16. A possible explanation for this may lie in the very precise structure of the virion itself. The surface of Sindbis is a T=4 icosahedral shell made rigid by scaffolding interaction among the E1 glycoproteins. In the mature virion, the E1 glycoprotein is a highly constrained energy-rich metastable structure. The energy stored in E1 is believed to be used to disassemble the protein lattice and to allow virus-cell membrane fusion. The constrained form of E1 is developed in the endoplasmic reticulum of infected cells by folding through several disulfide bridged intermediates as the PE2-E1 heterotrimer is produced. The energy rich form of E1 rapidly reorganizes to a lower energy state by the reshuffling of disulfide bridges if the protein is isolated from the virion in the absence of thiol blocking agents. It has also been demonstrated that the function of the membrane glycoprotein is affected by mutations in the core protein, suggesting that specific interactions between the capsid and the membrane protein E2 are critical to virus stability. The rigid organization of the virus membrane glycoproteins and the identical structure of the inner core may require the E2 endodomain (cytoplasmic location), which binds to the virus core via interactions with the capsid protein hydrophobic pocket, emerges from the membrane in a particular orientation. The correct orientation may be required for the very specific binding of the E2 endodomain to the hydrophobic cleft in the capsid protein. As amino acids are removed from the transmembrane domain helix, the orientation of the E2 tail may be altered at the point of egress from the membrane bilayer. Alternatively, deletions in the transmembrane domain of Sindbis E2 may distort the E2 ectodomain, the domain oriented toward the exterior of the cell, thereby destabilizing interactions with the scaffolding protein E1. In the case of TM 16, this may result in an association

that allows for virus assembly but which produces a relatively unstable virion. This instability may result in the spontaneous reshuffling of disulfide bridges in the E1 glycoprotein to a low energy, non-native state which is antigenically dissimilar from native protein and may cause the structural degradation of the virion. The E2 tail of the mutant TM 17 contains an additional amino acid in the transmembrane domain helix and is predicted to exit the membrane at a position 100.degree. distant to that of the TM 16 mutant. This may relieve sufficient structural strain to allow the mutant to remain stable and immunogenic.

In summary, differences in the structure and physical properties of insect and mammalian cells have been exploited to produce host range mutations with potential as vaccines. This approach should be applicable to the production of vaccines against any of the several hundred membrane-containing insect borne viruses for which a cDNA clone can be produced.

EXAMPLE 8

Properties and Uses of TM10 Mutant

The TM 10 mutant has 16 amino acids deleted from the viral transmembrane protein and there are only 10 amino acids left in the transmembrane domain. Ordinarily, a domain with 10 amino acids would not be expected to be able to span a membrane bilayer because a minimal of 14 amino acids is required. However, the TM10 mutant exhibits some unexpected properties that indicate to a person having ordinary skill in this art that TM10 may be useful in vaccine development.

Numerous deletions have been made reducing the transmembrane domain from its wild type length of 26 amino acids to as short as 10 amino acids. These deletions have pronounced effects on the production of infectious viruses in cells of the vertebrate and invertebrate hosts (FIG. 7). The production of infectious viruses followed a curve with minimal amount of viruses produced after a single deletion and a deletion of 14 amino acids. Surprisingly, deletion of 16 amino acids (TM10) restored virus production to high levels even though it has been demonstrated that a domain of 10 amino acids is too short to span a membrane bilayer (Adams and Rose 1985). The TM10 mutant was also relatively heat stable (FIG. 8) and more infectious (FIG. 9) compared to other transmembrane mutants.

All transmembrane mutants with 14 or more amino acids in the transmembrane domain produced proteins with a profile similar to that of wild type virus (FIG. 10). TM10 and TM12 in contrast produced aberrantly processed proteins in addition to the proteins seen in the wild type and larger transmembrane mutants. Analysis by mass spectrometry showed that the aberrant proteins seen in the TM10 mutant contained the sequence of protein E2 and the non-structural protein 6K, indicating that TM10 failed to proteolytically process the junction between these two proteins.

The ability of mutant TM10 to produce significant, albeit reduced, amount of viruses which are stable suggests that this mutant may produce an immune response in the absence of pathogenesis. This prediction is based on the observation that live virus vaccines frequently produce lower amount of viruses than the parental virulent forms. The large size of deletion in the TM10 mutant suggests that reversion to wild type phenotype is unlikely. The relative stability suggests that storage of this mutant may not result in significant loss of infectivity.

TM10 Animal Testing

The TM10 mutation has been installed in a highly pathogenic form of Sindbis virus, TR339. Three day old CD-1 mice will be injected with 1000 plaque forming units of TR339 or TR339TM10 or Buffer. At 14 days post vaccination surviving mice will be challenged with TR339 (1000 PFU). Morbidity and mortality is determined in the three sets. Mice receiving buffer should show high mortality and total morbidity after challenge with TR339. Mice receiving TR 339 will likely not survive vaccination. Mice receiving TR339 TM10 should survive vaccination with low morbidity and be completely resistant to challenge with TR339.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

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34rtificial sequenceUsed as the mutagenesis primer with the "forward primer" to generate a 5 "Megaprimer"corresponding to nucleotides 9295-98cacggcgc gcacaggcac ataacactgc 3artificial sequenceUsed as the "forward primer" with the mutagenesis primer to generate a 5 megaprimer corresponding to nucleotides 9295-98atcaagca gtgcgtcg Aartificial sequenceUsed as the "reverse primer" with the megaprimer and the Toto asmid template to create cleotide product used to create the deletion mutant K39to ggcagtgtgc accttaatcg cctgc 25426PRTSindbis virustransmembrane domain of E2 in the PE2sequence 365..39Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala Met Met 5 e Gly Val Thr Val Ala Val Leu Cys Ala Cys 2DNAartificial sequence93Forward primer Efrom megaprimer used with reverse primer to generate deletion constructs containing unique BclI and SplI sites. 5gcgtcgccta

taagagcgac c 2artificial sequenceerse primer Splext from megaprimer used with forward primer to generate deletion constructs containing unique BclI and SplI sites. 6cagtgtgcac cttaatcgcc tgc 23724DNAartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. 7acataacact gcgatggtgt acac 24823DNAartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. 8acataacact gcggctaaga tgg 23922DNAartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. 9acataacact gctgcgacgg ct 22artificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. agtta cgacggctaa g 2Aartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. tacgc cgacggctaa g 2Aartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. gccaa tgacggctaa g 2Aartificial sequenceMutagenic primer E2 TMative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. atcat gacggctaag a 2Aartificial sequenceMutagenic primer E2 TM2tive strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. agtta cggtagctga 2Aartificial sequenceMutagenic primer E2 TM2tive strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. cgccg gtagctga NAartificial sequenceMutagenic primer E2 TM22 (negative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. cagtt accgccacgg t 2Aartificial sequenceMutagenic primer E2 TM23 (negative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. tacgc ccgccacggt 2Aartificial sequenceMutagenic primer E2 TM24 (negative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. gccaa tcgccacggt 2Aartificial sequenceMutagenic primer E2 TM25 (negative strand) used to create a deletion in the E2 transmembranal domain in the Sindbis viral glycoprotein. aatca tcgccacggt 2ASindbis virus97tide sequence of the E2 transmembranal domain of the Sindbis viral glycoprotein 2gtgt acaccatctt agccgtcgca tcagctaccg tggcgatgat 5cgta actgttgcag tgttatgtgc ctgt 842indbis virus363..39acid sequence of the E2 transmembranal domain of the Sindbis viral glycoprotein 2o Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala 5 t Met Ile Gly Val Thr Val Ala Val Leu Cys Ala Cys 27PRTartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acid 378, the resulting deletion mutant is TM25. 22His Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala 5 t Ile Gly Val Thr Val Ala Val Leu Cys Ala Cys 26PRTartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 378 and 379, the resulting deletion mutant is TM24 23His Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala 5 e Gly Val Thr Val Ala Val Leu Cys Ala Cys 25PRTartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 378 through 38resulting deletion mutant is TM23 24His Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala 5 y Val Thr Val Ala Val Leu Cys Ala Cys 24PRTartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 378 through 38resulting deletion mutant is TM22. 25His Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Ala 5 l Thr Val Ala Val Leu Cys Ala Cys 2Tartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 376 through 38resulting deletion mutant is TM2s Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Gly Val 5 r Val Ala Val Leu Cys Ala Cys 2Tartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 376 through 38resulting deletion mutant is TM2s Pro Val Tyr Thr Ile Leu Ala Val Ala Ser Ala Thr Val Thr 5 l Ala Val Leu Cys Ala Cys 2Tartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 372 through 378, the resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Val Met Ile Gly Val Thr Val 5

a Val Leu Cys Ala Cys 2Tartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 372 through 379, the resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Val Ile Gly Val Thr Val Ala 5 l Leu Cys Ala Cys 2Tartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 372 through 38resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Val Gly Val Thr Val Ala Val 5 u Cys Ala Cys3rtificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 372 through 38resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Val Val Thr Val Ala Val Leu 5 s Ala Cys32tificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 373 through 384, the resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Val Ala Ala Val Leu Cys Ala 5 s33tificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 37gh 384, the resulting deletion mutant is TMis Pro Val Tyr Thr Ile Leu Ala Ala Val Leu Cys Ala Cys 5 RTartificial sequenceSequence of the E2 transmembranal domain of the Sindbis viral glycoprotein after deleting amino acids 369 through 384, the resulting deletion mutant is TMis Pro Val Tyr Thr Ile Ala Val Leu Cys Ala Cys 5

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United States Patent **7,335,471**
Guillerez , et al. **February 26, 2008**

Polypeptides derived from RNA polymerases and use thereof

Abstract

Mutant RNA polymerases of phagic origin in which the peptide chain is modified by substitution, deletion or addition of at least one amino acid, the modification having the effect of reducing the sensitivity of the RNA polymerases to the initial transcription sequence of the DNA sequence coding for the RNA, for a method for production of the RNA, or proteins coded by the RNA, from given nucleotide sequences, comprising a sequence of DNA coding for the RNA, the transcription of which is placed under the control of a promoter recognised by wild-type RNA polymerases and the mutant RNA polymerases as above. The method has a higher yield of RNA than the yield obtained when using the wild-type RNA polymerases in the presence of the same non-consensual ITS as that found in the sequence of DNA coding for the RNA.

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Current U.S. Class: **435/6** ; 435/91.3
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Claims

The invention claimed is:

1. A method for producing a RNA of interest, or proteins coded by this RNA of interest, comprising: synthesizing RNA with mutated RNA polymerases of phage origin, the peptide chain of which is modified, with respect to the ***wild-type*** RNA polymerases from which they originate, by substitution, or deletion, or addition of at least one amino acid, this modification having the effect of reducing the sensitivity of said RNA polymerases to the initial transcribed sequence (ITS) contained in a DNA sequence coding

for an RNA of interest, starting from determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned ***wild-type*** RNA polymerases and mutated RNA polymerases, said method having a production yield of said RNA greater than the yield obtained in the case of use of the ***wild-type*** RNA polymerases in the presence of the same non-consensual ITS as that contained in the DNA sequence coding for said RNA of interest, wherein said mutated RNA polymerases are selected from the group consisting of: those comprising the mutated T7 RNA polymerase represented by SEQ ID NO: 2, comprising a leucine in position 266 substituted for the proline in the ***wild-type*** T7 RNA polymerase, or of the proline situated in the homologous position in the ***wild-type*** RNA polymerases of other bacteriophages, selected from the group consisting of the proline situated in positions 267 in T3, 289 in KII, and 239 in SP6.

2. A method for producing a RNA of interest, or proteins coded by this RNA of interest, comprising: synthesizing RNA with mutated RNA polymerases of phage origin, the peptide chain of which is modified, with respect to the ***wild-type*** RNA polymerases from which they originate, by substitution, or deletion, or addition of at least one amino acid, this modification having the effect of reducing the sensitivity of said RNA polymerases to the ITS contained in a DNA sequence coding for an RNA of interest, starting from determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned ***wild-type*** RNA polymerases and mutated RNA polymerases, said method having a production yield of said RNA greater than the yield obtained in the case of use of the ***wild-type*** RNA polymerases in the presence of the same non-consensual ITS as that contained in the DNA sequence coding for said RNA of interest, wherein said mutated RNA polymerases are selected from the group consisting of: those derived from the ***wild-type*** T7 RNA polymerase, and comprising at least one of the following mutations: replacement of the isoleucine I) in position 117 by a valine (V), replacement of the isoleucine I) in position 119 by a valine (V), replacement of the valine (V) in position 134 by an alanine (A), replacement of the aspartic acid (D) in position 147 by asparagine (N), replacement of the histidine (H) in position 230 by an arginine (R), replacement of the proline (P) in position 266 by a leucine (L), replacement of the arginine (R) in position 291 by a cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 639 by a phenylalanine (F), replacement of the isoleucine (I) in position 810 by an asparagine (N), those derived from the ***wild-type*** T3 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 148 by asparagine (N), replacement of the proline (P) in position 267 by a leucine (L), replacement of the arginine (R) in position 292 by a cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 640 by a phenylalanine (F), replacement of the isoleucine (I in position 811 by an asparagine (N), those derived from the ***wild-type*** KII RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 167 by asparagine (N), replacement of the proline (P) in position 289 by a leucine (L), replacement of the arginine (R) in position 314 by a cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 662 by a phenylalanine (F), replacement of the isoleucine (I) in position 833 by an asparagine (N), and those derived from the ***wild-type*** SP6 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 117 by asparagine (N), replacement of the proline (P) in position 239 by a leucine (L), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 631 by a phenylalanine (F), replacement of the isoleucine (I) in position 804 by an asparagine (N).

3. A method for producing a RNA of interest, or proteins coded by this RNA of interest, comprising: synthesizing RNA with mutated RNA polymerases of phage origin, the peptide chain of which is modified, with respect to the ***wild-type*** RNA polymerases from which they originate, by substitution, or deletion, or addition of at least one amino acid, this modification having the effect of reducing the sensitivity of said RNA polymerases to the ITS contained in a DNA sequence coding for an RNA of interest, starting from determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned ***wild-type*** RNA polymerases and mutated RNA polymerases, said method having a production yield of said RNA greater than the yield obtained in the case of use of the ***wild-type*** RNA polymerases in the presence of the same non-consensual ITS as that contained in the DNA sequence coding for said RNA of interest, wherein said mutated RNA polymerases are selected from the group consisting of: the mutated T7 RNA polymerase represented by SEQ ID NO: 2, comprising a leucine in position 266 substituted for the proline, the mutated T7 RNA polymerase represented by SEQ ID NO: 4, comprising a valine in position 117 substituted for the isoleucine, and an alanine in position 134 substituted for the valine, the mutated T7 RNA polymerase represented by SEQ ID NO: 6, comprising a valine in position 119 substituted for the isoleucine, and an asparagine in position 147 substituted for the aspartic acid, the mutated T7 RNA polymerase represented by SEQ ID NO: 8, comprising an arginine in position 230 substituted for the histidine, and a cysteine in position 291 substituted for the arginine, the mutated T7 RNA polymerase represented by SEQ ID NO: 10, comprising a leucine in position 266 substituted for the proline, and a phenylalanine in position 639 substituted for the tyrosine, the mutated T7 RNA polymerase represented by SEQ ID NO: 12, comprising an asparagine in position 810 substituted for the isoleucine, the mutated T7 RNA polymerase represented by SEQ ID NO: 14, comprising a leucine in position 266 substituted for the proline, and an asparagine in position 810 substituted for the isoleucine, and the mutated T7 RNA polymerase represented by SEQ ID NO: 16, comprising a valine in position 119 substituted for the isoleucine, an asparagine in position 147 substituted for the aspartic acid, and an asparagine in position 810 substituted for the isoleucine.

Description

A subject of the present invention is polypeptides derived from RNA polymerases, also designated mutated RNA polymerases, as well as their uses, in particular in the field of in vivo or in vitro preparation of RNA of interest.

When obtaining large quantities of DNA molecules of perfectly defined sequence, high purity and small size (10 to 100 nt) chemical synthesis is at present the most economical method. There are now in the market a very large number of enterprises which carry out these syntheses on request. These same companies also offer the chemical synthesis of RNA but the technology is such that the cost, which is at least ten times higher than for DNA, is at present prohibitive.

Enzyme synthesis is therefore favoured, using an RNA polymerase coupled to a DNA matrix, chemically synthesized or of plasmid origin, which comprises the sequence to be transcribed downstream of the promoter sequence of the polymerase used. The necessary elements (polymerases, rNTP, cloning vectors) are available on the market in the form of optimized kits.

Bacteriophage T7 RNA polymerase is very widely used for carrying out transcription reactions in vitro with the aim of synthesizing large quantities (up to a milligram) of RNA from matrices of recombinant DNA. These RNAs synthesized in vitro are necessary for a number of uses in Biology, the Biotechnologies and Pharmacy; the following can be mentioned inter alia: translation in vitro. the study of RNA maturation. the effect of anti-sense RNA on gene expression. RNA-protein interactions. the synthesis of homologues of small cell RNAs (tRNA, rRNA). the production of ribozymes.

More generally these RNAs are also used as molecular probes and structural study substrates.

A few T7 polymerase mutants have already been described. These are mutated RNA polymerases derived from the wild-type T7 RNA polymerase, and comprising one of the following mutations: replacement of the lysine (K) in position 222 by glutamic acid; this RNA polymerase thus mutated has the property of recognizing a promoter altered by mutation, and is described in U.S. Pat. No. 5,385,834, replacement of the tyrosine (Y) in position 639 by a phenylalanine (F); this RNA polymerase thus mutated has the property of incorporating deoxyribonucleotides instead of ribonucleotides, thus allowing the synthesis of DNA instead of RNA, and is described in U.S. Pat. No. 6,107,037, replacement of the isoleucine (I) in position 810 by a serine (S); this RNA polymerase thus mutated has the property of being slower than the wild-type RNA polymerase, and is described in Bonner et al., The Journal of Biochemical Chemistry, 269, pp. 25120-25128 (1994).

The problem to be resolved by the present invention, is linked with the fact that the activity on a given DNA matrix of the T7 RNA polymerase (and moreover of all the other known RNA polymerases) is strongly dependent on the nature of the first 6-12 nucleotides transcribed (Initial Transcribed Sequence, or ITS) of the nucleotide sequence coding for a given RNA (Milligan et al., 1987). If the ITS differs too much from the consensus sequence 5'GGGAGA . . . then the polymerase frequently aborts and a large quantity of the ribonucleoside triphosphates is consumed in order to synthesize small abortive RNAs to the detriment of the desired large RNAs.

Therefore, the experimenter is generally compelled, in order to obtain an effective transcription, to modify the sequence that is to be transcribed, in order to add to it a favourable ITS (also referred to as consensus or consensual ITS). In a number of cases, this constraint is very annoying, even unacceptable.

A potential method for obtaining any non-consensual 5' end RNA uses an after treatment of an RNA comprising the desired sequence downstream of a consensus ITS; the latter (target) RNA can therefore be produced in abundance. Moreover it is necessary to chemically synthesize a chimeric DNA-RNA oligonucleotide the DNA sequence of which is complementary to the 3' end of the RNA to be eliminated, then the hybrid that can form the oligo with the target transcript is digested by RNase H. This complex and onerous method is justified if the desired RNA is of a very large size and if its 5' end must be rigorously defined. (Li et al. 1999, Lapham et al. 1997).

The addition, to the reaction medium, of synthetic polyamines can increase the production of RNA by, it appears, reducing the number of abortive cycles; however determination of the optimum experimental conditions (choice of polyamine, concentration of use) requires case-by-case research. Moreover it should be noted that the effect of these polyamines is weak, even non-existent, when the matrix is of plasmid origin (double strand) (Frugier et al. 1994).

These two methods use reagents which are not in common use (specific polyamines or oligonucleotides) and are unsuitable for routine use. It is evident that the possibility of having, at the start, a polymerase which by nature aborts less would be a much simpler way of resolving the difficulty.

The invention results from the demonstration by the Inventors of the fact that modifying the peptide sequence of the RNA polymerases could reduce the sensitivity of the RNA polymerases thus modified to the nature of the ITS of the nucleotide sequences to be transcribed.

The invention aims to provide polypeptides derived from wild-type RNA polymerases usually used in the production of RNA of interest, which are distinctly less sensitive to the nature of the non-consensual ITS than said wild-type RNA polymerases.

The invention also aims to provide new processes for producing RNA of interest with yields greater than those of the processes using wild-type RNA polymerases when the latter are sensitive to the ITS of the nucleotide sequence coding for said RNA of interest.

A subject of the invention is the use of mutated RNA polymerases of phage origin, namely of RNA polymerases originating from phages, the peptide chain of which is modified, with respect to the wild-type RNA polymerases from which they derive, by substitution, or deletion, or addition of at least one amino acid, this modification having the effect of reducing the sensitivity of said RNA polymerases to the ITS contained in the DNA sequence coding for an RNA of interest, for the implementation of a process for producing said RNA of interest, or of proteins coded by this RNA of interest, starting with determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned wild-type RNA polymerases and mutated RNA polymerases, said process having a production yield of said RNA greater than the yield obtained in the case of use of the wild-type RNA polymerases (in the presence of the same non-consensual ITS as that contained in the DNA sequence coding for said RNA of interest).

The activity of said RNA polymerases thus mutated within the transcription of the DNA sequence coding for said RNA of interest is distinctly less affected by the nature of the nucleotides constituting the non-consensual ITS of this DNA sequence, which is not that of the wild-type RNA polymerases from which they derive, which allows these mutated RNA polymerases to be up to approximately 40 times more active than the wild-type polymerases, and therefore, in the abovementioned process of the present invention, to be characterized by a production yield of said RNA greater than the yield obtained in the case of use of the wild-type RNA polymerases in the presence of this same non-consensual ITS.

According to another particularly advantageous aspect of the invention, the mutated RNA polymerases of the invention make it possible to obtain RNA of interest with a practically identical yield, whatever the ITS present in the sequence coding for said RNA of interest.

Advantageously, the use of the abovementioned mutated RNA polymerases allows the implementation of a process for producing RNA of interest, the yield of which is up to approximately ten times greater than the yield obtained in the case of use of the wild-type RNA polymerases in the presence of a non-consensual ITS.

The RNA of interest capable of being produced in greater quantity within the implementation of a process according to the invention using the abovementioned mutated RNA polymerases, are natural or chimeric RNAs, said RNAs if appropriate comprising one or more non-canonical nucleoside monophosphates (namely for example a deoxyribose instead of a ribose, said sugar itself being able optionally to carry an analogue of one of the natural nucleic bases if this analogue is recognized as such by the polymerase). In the latter case, a subject of the present invention is also the use of the above-mentioned process using said above-mentioned RNA polymerases, in the implementation of a method for determining the sequence of a nucleic acid molecule.

The mutated RNA polymerases used are advantageously those deriving from wild-type phage monomeric polymerases, in particular those originating from monomeric RNA polymerases of bacteriophages such as T7, T3, K11, SP6, respectively described in particular in the article by W. T. McAllister and C. A. Raskin, Molecular Microbiology (1993), 10(1), 1-6.

Preferably the abovementioned mutated RNA polymerases are those deriving from the wild-type RNA polymerases at least one of the amino acids of which, situated between positions 1 and approximately 410, in particular approximately between positions 90 and 320, more particularly between positions 115 and 300, is modified by substitution or deletion.

Advantageously, the mutated RNA polymerases used are those comprising a leucine in position 266, substituted for the proline situated in position 266 in the wild-type T7 RNA polymerase, or the proline situated in homologous position in the wild-type RNA polymerases of bacteriophages, such as the proline situated in positions 267 in T3, 289 in K11, and 239 in SP6.

A more particular subject of the invention is the abovementioned use of any mutated RNA polymerase as defined above, the proline of which, defined above, and/or at least one of the amino acids situated in the vicinity of the abovementioned proline, namely an amino acid situated at a distance less than or equal to approximately 10 angstroms from the proline in question, when said RNA polymerase is considered in its three-dimensional structure (as described in Cheetham, G. M. & Steitz, T. A. (1999), Science 286, 2305-2309; Cheetham, G. M. et al., Nature 399, 80-83; Sousa, R. et al., Nature 364, 593-599), is modified by substitution or deletion.

The invention also relates to the abovementioned use of the particularly preferred mutated RNA polymerases chosen from the following: those derived from the wild-type T7 RNA polymerase, and comprising at least one of the following mutations: replacement of the isoleucine (I) in position 117 by a valine (V), replacement of the isoleucine (I) in position 119 by a valine (V), replacement of the valine (V) in position 134 by an alanine (A), replacement of the aspartic acid (D) in position 147 by asparagine (N), replacement of the histidine (H) in position 230 by an arginine (R), replacement of the proline (P) in position 266 by a leucine (L), replacement of the arginine (R) in position 291 by a cysteine (C), those derived from the wild-type T3 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 148 by asparagine (N), replacement of the proline (P) in position 267 by a leucine (L), replacement of the arginine (R) in position 292 by a cysteine (C), those derived from the wild-type K11 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 167 by asparagine (N), replacement of the proline (P) in position 289 by a leucine (L), replacement of the arginine (R) in position 314 by a cysteine (C), those derived from the wild-type SP6 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 117 by asparagine (N), replacement of the proline (P) in position 239 by a leucine (L).

A more particular subject of the invention is the abovementioned use of the following mutated RNA polymerases: the mutated T7 RNA polymerase represented by SEQ ID NO: 2, comprising a leucine in position 266 substituted for the proline, the mutated T7 RNA polymerase represented by SEQ ID NO: 4, comprising a valine in position 117

substituted for the isoleucine, and an alanine in position 134 substituted for the valine, the mutated T7 RNA polymerase represented by SEQ ID NO: 6, comprising a valine in position 119 substituted for the isoleucine, and an asparagine in position 147 substituted for the aspartic acid, the mutated T7 RNA polymerase represented by SEQ ID NO: 8, comprising an arginine in position 230 substituted for the histidine, and a cysteine in position 291 substituted for the arginine, the mutated T7 RNA polymerase represented by SEQ ID NO: 10, comprising a leucine in position 266 substituted for the proline, and a phenylalanine in position 639 substituted for the tyrosine, the mutated T7 RNA polymerase represented by SEQ ID NO: 12, comprising an asparagine in position 810 substituted for the isoleucine, the mutated T7 RNA polymerase represented by SEQ ID NO: 14, comprising a leucine in position 266 substituted for the proline, and an asparagine in position 810 substituted for the isoleucine, the mutated T7 RNA polymerase represented by SEQ ID NO: 16, comprising a valine in position 119 substituted for the isoleucine, an asparagine in position 147 substituted for the aspartic acid, and an asparagine in position 810 substituted for the isoleucine.

A subject of the invention is also the use described above of abovementioned mutated RNA polymerases comprising as well as the modification(s) of amino acid(s) defined above, the modification, by substitution, or deletion, or addition, of at least one other amino acid involved in a mechanism other than that of sensitivity to the ITS within the scope of the transcription of DNA sequences, such as a modification of an amino acid making it possible for the RNA polymerase thus modified to incorporate non-canonical nucleoside triphosphates (for example dNTP) in an RNA chain, and therefore making it possible to synthesize DNA, and/or a modification of an amino acid making it possible to obtain a slower RNA polymerase, and therefore, in association with a mutation of the invention, making it possible to improve the synthesis yields of proteins of interest.

A more particular subject of the invention is therefore the abovementioned use of mutated RNA polymerases as defined above, comprising as well as the mutation(s) defined above, a mutation corresponding to the substitution of the tyrosine in position 639 in T7, or in analogous position in the other polymerases defined above, by a phenylalanine, and/or a mutation corresponding to the substitution of the isoleucine in position 810 in T7, or in analogous position in the other polymerases defined above, by a serine, or by an asparagine.

Therefore, the invention also relates more particularly to the use of the abovementioned mutated T7 RNA polymerase represented by SEQ ID NO: 10.

A subject of the invention is also: the mutated RNA polymerases derived from the wild-type T7 RNA polymerase comprising at least one of the abovementioned mutations in position 117, 119, 134, 147, 230, 266, or 291, and optionally at least one of the following additional mutations: replacement of the tyrosine (Y) in position 639 by a phenylalanine (F), replacement of the isoleucine (I) in position 810 by an asparagine (N), the mutated RNA polymerases derived from the wild-type T3 RNA polymerase comprising at least one of the abovementioned mutations in position 148, 267, or 292, and optionally at least one of the following additional mutations: replacement of the tyrosine (Y) in position 640 by a phenylalanine (F), replacement of the isoleucine (I) in position 811 by an asparagine (N), the mutated RNA polymerases derived from the wild-type K11 RNA polymerase comprising at least one of the abovementioned mutations in position 167, 289, or 314, and optionally at least one of the following additional mutations: replacement of the tyrosine (Y) in position 662 by a phenylalanine (F), replacement of the isoleucine (I) in position 833 by an asparagine (N), the mutated RNA polymerases derived from the wild-type SP6 RNA polymerase comprising at least one of the abovementioned mutations in position 117 or 239, and optionally at least one of the following additional mutations: replacement of the tyrosine (Y) in position 631 by a phenylalanine (F), replacement of the isoleucine (I) in position 804 by an asparagine (N).

The invention also relates to any process for preparing mutated RNA polymerases of phage origin as defined above, namely of RNA polymerases, the determined nucleotide sequence transcription activity of which, comprising a DNA sequence coding for an RNA of interest and the transcription of which is placed under the control of a promoter recognized by the mutated RNA polymerases and by the wild-type RNA polymerases of phage origin from which they originate, is greater than the transcription activity of this determined sequence by the wild-type RNA polymerases, in particular preparation of mutated RNA polymerases being up to approximately 40 times more active than said wild-type RNA polymerases within the scope of the implementation of processes for producing RNA of interest starting with said determined nucleotide sequence, said process comprising: a stage of modification of the peptide chain of the wild-type RNA polymerases of phage origin by substitution, or deletion or addition of at least one codon of the gene coding for said wild-type RNA polymerases, and transformation of appropriate cells, such as E. coli, with vectors containing the gene thus modified, detection of the abovementioned cells producing mutated RNA polymerases for which the production yield of a particular marker within appropriate cells, such as E. coli, in particular within the abovementioned cells, such as a marker of resistance to an antibiotic, or a chromogenic marker, coded by a nucleotide sequence inserted downstream of the promoter recognized by the abovementioned RNA polymerases, this promoter and the sequence coding for the marker being separated by an ITS, the nature of approximately the first 6 to 12 nucleotides of which is known to affect the activity of the wild-type RNA polymerases, is greater than the production yield of this same marker obtained by use of the wild-type RNA polymerases under the same conditions, purification of the abovementioned mutated RNA polymerases from the cells detected in the preceding stage.

A more particular subject of the invention is the abovementioned use of mutated RNA polymerases of phage origin as obtained by implementation of the preparation process defined above.

The invention also relates to mutated RNA polymerases of phage origin as obtained by implementation of a preparation process defined above.

A more particular subject of the invention is the mutated RNA polymerases of phage origin as obtained by implementation of a preparation process defined above, originating from the modification of wild-type phage monomeric polymerases, in particular originating from monomeric RNA polymerases of bacteriophages such as T7, T3, K11, SP6.

A subject of the invention is also the abovementioned mutated RNA polymerases of phage origin deriving from the RNA polymerases of wild-type phage origin, at least one of the amino acids of which, situated between positions 1 and approximately 410, in particular approximately between positions 90 and 320, more particularly between positions 115 and 300, is modified by substitution or deletion, to the exclusion of the mutated RNA polymerase derived from the wild-type T7 RNA polymerase, and comprising the following mutation: replacement of the lysine (K) in position 222 by glutamic acid.

A more particular subject of the invention is the abovementioned mutated RNA polymerases comprising a leucine in position 266, substituted for the proline in position 266 in the wild-type T7 RNA polymerase, or in the homologous positions in the wild-type bacteriophage RNA polymerases, such as positions 267 in T3, 289 in K11, and 239 in SP6.

A more particular subject of the invention is also any mutated RNA polymerase as defined above, of which the proline defined above, and/or at least one of the amino acids situated in the vicinity of the abovementioned proline, namely an amino acid situated at a distance less than or equal to approximately 10 angstroms from the proline in question, when said RNA polymerase is considered in its three-dimensional structure, is modified by substitution or deletion.

The invention more particularly relates to the mutated RNA polymerases derived from the wild-type RNA polymerases such as T7, T3, K11 or SP6, and chosen from the following: those derived from the wild-type T7 RNA polymerase, and comprising at least one of the following mutations: replacement of the isoleucine (I) in position 117 by a valine (V), replacement of the isoleucine (I) in position 119 by a valine (V), replacement of the valine (V) in position 134 by an alanine (A), replacement of the aspartic acid (D) in position 147 by asparagine (N), replacement of the histidine (H) in position 230 by an arginine (R), replacement of the proline (P) in position 266 by a leucine (L), replacement of the arginine (R) in position 291 by a cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 639 by a phenylalanine (F), replacement of the isoleucine (I) in position 811 by an asparagine (N), those derived from the wild-type T3 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 148 by asparagine (N), replacement of the proline (P) in position 267 by a leucine (L), replacement of the arginine (R) in position 292 by a cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 640 by a phenylalanine (F), replacement of the isoleucine (I) in position 811 by an asparagine (N), those derived from the wild-type K11 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 167 by asparagine (N), replacement of the proline (P) in position 289 by a leucine (L), replacement of the arginine (R) in position 314 by a

cysteine (C), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 662 by a phenylalanine (F), replacement of the isoleucine (I) in position 833 by an asparagine (N), those derived from the wild-type SP6 RNA polymerase, and comprising at least one of the following mutations: replacement of the aspartic acid (D) in position 117 by asparagine (N), replacement of the proline (P) in position 239 by a leucine (L), said mutated RNA polymerases optionally comprising at least one of the following additional mutations: replacement of the tyrosine (Y) in position 631 by a phenylalanine (F), replacement of the isoleucine (I) in position 804 by an asparagine (N).

A more particular subject of the invention is the following mutated RNA polymerases: the mutated T7 RNA polymerase represented by SEQ ID NO: 2, comprising a leucine in position 266 substituted for the proline, the mutated T7 RNA polymerase represented by SEQ ID NO: 4, comprising a valine in position 117 substituted for the isoleucine, and an alanine in position 134 substituted for the valine, the mutated T7 RNA polymerase represented by SEQ ID NO: 6, comprising a valine in position 119 substituted for the isoleucine, and an asparagine in position 147 substituted for the aspartic acid, the mutated T7 RNA polymerase represented by SEQ ID NO: 8, comprising an arginine in position 230 substituted for the histidine, and a cysteine in position 291 substituted for the arginine, the mutated T7 RNA polymerase represented by SEQ ID NO: 10, comprising a leucine in position 266 substituted for the proline, and a phenylalanine in position 639 substituted for the tyrosine, the mutated T7 RNA polymerase represented by SEQ ID NO: 12, comprising an asparagine in position 810 substituted for the isoleucine, the mutated T7 RNA polymerase represented by SEQ ID NO: 14, comprising a leucine in position 266 substituted for the proline, and an asparagine in position 810 substituted for the isoleucine, the mutated T7 RNA polymerase represented by SEQ ID NO: 16, comprising a valine in position 119 substituted for the isoleucine, an asparagine in position 147 substituted for the aspartic acid, and an asparagine in position 810 substituted for the isoleucine.

The invention also relates to the nucleotide sequences coding for a mutated RNA polymerase of phage origin as defined above.

Therefore a more particular subject of the invention is the nucleotide sequences SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, coding respectively for the abovementioned proteins SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, or any nucleotide sequence derived from the abovementioned nucleotide sequences by degeneration of the genetic code and retaining the property of coding for the abovementioned proteins.

A subject of the invention is also any vector, in particular any plasmid, containing a nucleotide sequence as defined above.

A subject of the invention is also any cell transformed by an abovementioned vector, said cell being chosen in particular from those of bacteria (E. coli for example), yeasts, or higher eukaryotes.

The invention also relates to any process for preparing the abovementioned mutated RNA polymerases of phage origin, comprising the culture of transformed cells defined above in an appropriate culture medium, and the purification of the RNA polymerases produced by said cells.

A subject of the invention is also any process for preparing in vitro RNA of interest, comprising the bringing together, in an appropriate medium, of at least one mutated RNA polymerase of phage origin as defined above, with the determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned wild-type RNA polymerases and mutated RNA polymerases.

The invention also relates to any process for preparing in vivo RNA of interest, comprising the culture of cells as defined above, said cells producing at least one mutated RNA polymerase of phage origin according to the invention, and the genome of which has been modified in order to contain determined nucleotide sequences comprising a DNA sequence coding for said RNA of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned wild-type RNA polymerases and mutated RNA polymerases.

A subject of the invention is also any process for preparing in vivo proteins of interest comprising the culture of cells as defined above, said cells producing at least one mutated RNA polymerase of phage origin according to the invention, and the genome of which has been modified in order to contain determined nucleotide sequences comprising a DNA sequence coding for said proteins of interest and the transcription of which is placed under the control of a promoter recognized by the abovementioned wild-type RNA polymerases and mutated RNA polymerases.

Advantageously, in the case of the implementation of processes for preparing proteins of interest as described above, the mutated RNA polymerases produced by the cells, are chosen from SEQ ID NO: 12, 14 and 16.

The invention also relates to any process for preparing in vitro or in vivo RNA or proteins of interest, as described above, and comprising, moreover, a stage of addition of synthetic polyamines as described by Frugier et al. Amongst the polyamines capable of being used, the following can be mentioned:
NH.sub.2(CH.sub.2).sub.3NH(CH.sub.2).sub.10NH(CH.sub.2).sub.3NH.sub.2 NH.sub.2(CH.sub.2).sub.3NH(CH.sub.2).sub.3NH(CH.sub.2).sub.10NH(CH.sub.2)-.sub.3NH(CH.sub.2).sub.3NH.sub.2

The invention is further illustrated using the following detailed description of the production of mutated RNA polymerases of phage origin according to the invention, and of their use for increasing the production of determined RNAs with respect to the production of these same RNAs using wild-type RNA polymerases.

Whilst the RNA polymerase of the bacteriophage T7 is normally very processive in the elongation phase, this is not the case during the transcription of the ITS: in this case, there is a high probability that the nascent transcript will be prematurely released (abortive transcription) (Martin et al., 1988). This is the number of abortive cycles that the enzyme must achieve, on average, before successfully transcribing the ITS and entering the elongation phase, which determines the frequency with which the complete transcripts are synthesized (Ikeda, 1992). This number is minimal when the ITS corresponds to the 5' sequence of the class III genes of the bacteriophage T7 (`consensual' ITS: 5' GGGAGA . . .). However, even under these conditions, the abortive transcripts make up a large proportion (40% to 60%) of the total transcripts. This proportion increases very rapidly when the ITS is removed from the consensus, or when the RNA polymerase carries mutations which reduce its rate of elongation, increasing the time necessary for the crossing of the ITS (Bonner et al., 1994; Bonner et al., 1992). Optionally, the combination of a non-consensual ITS and a slow polymerase leads to a situation where the enzyme loops indefinitely in the abortive phase: no large transcript is then synthesized. This property can be observed not only in vitro, but also in vivo, in cells of Escherichia coli expressing T7 RNA polymerase (Lopez et al., 1997; Makarova et al., 1995). It is the latter situation that we have exploited in order to select mutants of T7 RNA polymerase, the activity of which is less sensitive to the nature of the ITS than that of the wild-type enzyme.

We placed a target gene, the expression of which is easily detectable--the lacZ gene, the product of which is .beta.-galactosidase--preceded by a non-consensual ITS, under the control of the T7 promoter. This gene is then introduced into a bacterial cell containing moreover a plasmid coding for a T7 polymerase mutated in its catalytic site (i.e. a `slow' polymerase). As indicated above, the system, blocked in abortive phase, does not produce large transcripts, and therefore no .beta.-galactosidase. However, after random mutagenesis of the plasmid, it is possible to select bacterial clones which synthesize new mutants of the enzyme which are capable of expressing the target gene. Apart from the reverse mutants of the initial mutation of the catalytic site, we thus isolated mutations in the N-terminal part of the enzyme, which is not involved in the catalysis. When these new mutations were introduced into the wild-type enzyme, i.e. without mutations at the catalytic site, we observed that they reduced the susceptibility of the enzyme to the exact nature of the ITS. This is the point mutant P266L which is more particularly described here and forms the subject of the present invention. In fact, it makes it possible to transcribe, at usable levels, matrices on which the wild-type enzyme is practically inoperative.

DETAILED DESCRIPTION

a) Preparation, Purification and Storage

The point mutant SEQ ID NO: 2 obtained corresponds to the change in the sequence of the T7 polymerase of Proline 266 to Leucine (P266L). The gene of this mutated T7

polymerase is included in the pAR1219 plasmid under the control of an inducible promoter (pLac UV5). The protein (polymerase) is over-expressed in the BL 21 (ompF-) strain of E. coli. The protocols for extraction, purification and storage of the purified protein are identical to those of the wild-type polymerase. In order to facilitate the purification, the polymerase was labelled with six N-terminal histidines by transfer of the mutated region in place of the equivalent coding sequence of the pBH 161 plasmid. (He et al. 1977). This label in no way modifies the catalytic properties of the polymerase. It is then sufficient to pass the bacterial supernatant over a Nickel affinity column in order to obtain >90% pure protein. Storage for a long duration (2 years) at -20.degree. C. does not alter the properties of the enzyme.

b) Catalytic Activity.

Under the standard conditions used for the wild-type polymerase, the mutant is also capable of converting all the rNTPs provided to it to RNA. However the rate at which this incorporation is carried out is approximately three times lower for the mutant, i.e. a complete transcription will require a longer period of time (e.g. 6 hours instead of 2 hours).

As referred to below, the main distinction between the wild-type polymerase and the mutant is to be found at the level of distribution as a function of the size (and therefore of the mass) of the RNAs obtained. Two types of RNA can be observed on completion of in vitro transcription: abortive RNAs and large RNAs.

We present below the in vitro transcription results obtained with the wild-type polymerase and the mutant on seven very different ITSs. The matrices are plasmid DNAs (approximately 6 kbp) linearized at restriction sites situated from 31 to 78 nt downstream of the promoter. The reactions are stopped at 10, 20 or 30 minutes depending on the case, and the marking is .alpha..sup.32PGTP, .alpha..sup.32PUTP or .gamma..sup.32PGTP. The experimental conditions used are described in J. Mol. Biol. (1997) 269:41-51. The transcripts are separated on a high resolution denaturing polyacrylamide gel.

G10 (5' GGGAGACCA . . . linearized at 33 nt) carries the consensual ITS. In fact in this case the first 30 nucleotides correspond to gene 10 sequence of the phage T7, one of those better transcribed by T7 polymerase.

GGLac (5' GGGGAAUU. linearized at 69 nt) carries the ITS that is found in the commercial plasmid pET15b (Novagen) which comprises the binding site of the Lac repressor in order to optionally repress the T7 transcription.

Lac (5' GGAAUUG. linearized at 30 nt) is a plasmid equivalent to the preceding one, in which the operative site is close to 2 nucleotides of the promoter.

TyrG (5' GUCUCGG. linearized at 78 nt), Gly (5' ACUCUUU. linearized at 71 nt), Tyr (5' CUCUCGG. linearized at 78 nt) and Val (5' GGUUUCG. linearized at 31 nt) are plasmids corresponding to matrices intended to synthesize in vitro tRNAs or fragments of tRNA and deemed difficult to transcribe (Frugier et al., 1994)

FIGS. 1 to 7 show the detailed results indicating the abortive RNAs observed, their percentage with respect to the total number of transcripts, as well as the equivalent figures for the long transcripts which escaped the abortive phase, in the case of use of wild-type T7 polymerase (also designated wt, and represented using black columns) and of the use of the mutated RNA polymerase SEQ ID NO: 2 (also designated P266L, and represented using grey columns) in the presence of the ITS G10 (FIG. 1), GGLac (FIG. 2), TyrG (FIG. 3), Lac (FIG. 4), Gly (FIG. 5), Tyr (FIG. 6), Val (FIG. 7). The abortive transcripts are distributed between the 2 mer and the 13 mer (numbering 2 to 13 along the x-axis). The transcripts originating from polymerases which have escaped the abortive phase are identified as long transcripts (LT along the x-axis). A logarithmic scale is used along the y-axis in order to make it possible to quantify transcripts which are not very abundant.

These results show clearly that in all cases the mutant has less difficulty than the wild-type polymerase in incorporating the fifth, sixth and seventh nucleotides. If at these positions the polymerase has and/or must incorporate a pyrimidine (U or C) the abortion rate is raised for the wild-type polymerase and the removal of the handicap by the mutant is all the greater.

The immediate consequence is summarized in FIG. 8 which includes the results referred to previously, providing figures comparing the productivities of the two polymerases for increasingly unfavourable ITSs. The values indicated correspond to the percentage of incorporation of the rNTP into large transcripts in the case of use of the wild-type T7 polymerase (wt, black columns) and the use of the mutated RNA polymerase SEQ ID NO: 2 (P266L, grey columns).

Apart from G10 and GGLac where the two polymerases give complete satisfaction, in all other cases the wild-type polymerase has a low yield, even a very low yield, comprised between 22% and 1%, whilst that of the mutant remains reasonable, being comprised between 30% and 85%.

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52 DNA Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase ac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ctg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct tac ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg att cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88ccg gct gac gct gcg aac ctg ttc aaa gca gtg cgc gaa act atg 2496 Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823ac aca tat gag tct tgt gat gta ctg gct gat ttc tac gac cag 2544 Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84tc gct gac cag ttg cac gag tct caa ttg gac aaa atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala 2 883 PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase 2 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234lu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25rg Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28sn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33al Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345la Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36et Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378rg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 Asn Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534he Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556eu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57ys Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589al Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6Val Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662hr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634ys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ro Ala 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Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac gtc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Val Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gct cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Ala Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct

ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ccg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att 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artificial sequence mutated sequences of bacteriophage T7 RNA polymerase 5 atg aac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc gtt aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Val Lys Thr Thr Leu Ala Cys Leu

Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag aac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asn Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ccg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc

atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct tac ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg att cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88ccg gct gac gct gcg aac ctg ttc aaa gca gtg cgc gaa act atg 2496 Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823ac aca tat gag tct tgt gat gta ctg gct gat ttc tac gac cag 2544 Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84tc gct gac cag ttg cac gag tct caa ttg gac aaa atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala 6 883 PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase 6 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Val Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asn Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234lu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25rg Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28sn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33al Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345la Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36et Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378rg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 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Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76le Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778ln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823sp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84he Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878he Ala 7 2652 DNA Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase 7 atg aac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser >

aag ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cgc cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu Arg Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ccg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt tgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Cys Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr 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aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg att cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly 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atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala 8 883 PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase 8 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala 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Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 Asn Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534he Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556eu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57ys Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589al Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6Val Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662hr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634ys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr 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sequence mutated sequences of bacteriophage T7 RNA polymerase 9 atg aac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt

gca ggt gcg ctg gct ggc atc tct ctg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct ttc ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Phe Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg att cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88ccg gct gac gct gcg aac ctg ttc aaa gca gtg cgc gaa act atg 2496 Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823ac aca tat gag tct tgt gat gta ctg gct gat ttc tac gac cag 2544 Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84tc gct gac cag ttg cac gag tct caa ttg gac aaa atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234lu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25rg Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28sn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33al Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345la Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36et Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378rg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys

465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 Asn Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534he Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556eu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57ys Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589al Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6Val Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662hr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Phe Gly 625 634ys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ro Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667la Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68al Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77Lys Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76le Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778ln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823sp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84he Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878he Ala DNA Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase aac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ccg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc

gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct tac ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg aat cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Asn His Asp Ser Phe Gly Thr 88ccg gct gac gct gcg aac ctg ttc aaa gca gtg cgc gaa act atg 2496 Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823ac aca tat gag tct tgt gat gta ctg gct gat ttc tac gac cag 2544 Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84tc gct gac cag ttg cac gag tct caa ttg gac aaa atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser Gly Leu Leu Gly Gly Glu Ala Trp Ser Trp His Lys Glu Asp 2Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234lu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Gln Ala Ile Ala Thr 245 25rg Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28sn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33al Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345la Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36et Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378rg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 Asn Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534he Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556eu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57ys Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589al Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6Val Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662hr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634ys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ro Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667la Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68al Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77Lys Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76le Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778ln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr 88Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823sp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84he Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878he Ala DNA Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase aac acg att aac atc gct aag aac gac ttc tct gac atc gaa ctg 48 Met Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu gct atc ccg ttc aac act ctg gct gac cat tac ggt gag cgt tta 96 Ala Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct

ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc att aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag gac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ctg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct

cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct tac ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile 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Ala Asp His Tyr Gly Glu Arg Leu 2 gct cgc gaa cag ttg gcc ctt gag cat gag tct tac gag atg ggt gaa Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a cgc ttc cgc aag atg ttt gag cgt caa ctt aaa gct ggt gag gtt Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 gcg gat aac gct gcc gcc aag cct ctc atc act acc cta ctc cct aag 24sp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 atg att gca cgc atc aac gac tgg ttt gag gaa gtg aaa gct aag cgc 288 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9c aag cgc ccg aca gcc ttc cag ttc ctg caa gaa atc aag ccg gaa 336 Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu gta gcg tac atc acc gtt aag acc act ctg gct tgc cta acc agt 384 Ala Val Ala Tyr Ile Thr Val Lys Thr Thr Leu Ala Cys Leu Thr Ser gac aat aca acc gtt cag gct gta gca agc gca atc ggt cgg gcc 432 Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala gag aac gag gct cgc ttc ggt cgt atc cgt gac ctt gaa gct aag 48lu Asn Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys cac ttc aag aaa aac gtt gag gaa caa ctc aac aag cgc gta ggg cac 528 His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His tac aag aaa gca ttt atg caa gtt gtc gag gct gac atg ctc tct 576 Val Tyr Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser ggt cta ctc ggt ggc gag gcg tgg tct tcg tgg cat aag gaa gac 624 Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2att cat gta gga gta cgc tgc atc gag atg ctc att gag tca acc 672 Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222tg gtt agc tta cac cgc caa aat gct ggc gta gta ggt caa gac 72et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234ag act atc gaa ctc gca cct gaa tac gct gag gct atc gca acc 768 Ser Glu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25gt gca ggt gcg ctg gct ggc atc tct ctg atg ttc caa cct tgc gta 8Ala Gly Ala Leu Ala Gly Ile Ser Leu Met Phe Gln Pro Cys Val 267ct cct aag ccg tgg act ggc att act ggt ggt ggc tat tgg gct 864 Val Pro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28ac ggt cgt cgt cct ctg gcg ctg gtg cgt act cac agt aag aaa gca 9Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29atg cgc tac gaa gac gtt tac atg cct gag gtg tac aaa gcg att 96et Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33aac att gcg caa aac acc gca tgg aaa atc aac aag aaa gtc cta gcg n Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33tc gcc aac gta atc acc aag tgg aag cat tgt ccg gtc gag gac atc l Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345cg att gag cgt gaa gaa ctc ccg atg aaa ccg gaa gac atc gac o Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro

Glu Asp Ile Asp 355 36tg aat cct gag gct ctc acc gcg tgg aaa cgt gct gcc gct gct gtg t Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378gc aag gac aag gct cgc aag tct cgc cgt atc agc ctt gag ttc r Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39ctt gag caa gcc aat aag ttt gct aac cat aag gcc atc tgg ttc t Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44tac aac atg gac tgg cgc ggt cgt gtt tac gct gtg tca atg ttc o Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423cg caa ggt aac gat atg acc aaa gga ctg ctt acg ctg gcg aaa n Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44gt aaa cca atc ggt aag gaa

ggt tac tac tgg ctg aaa atc cac ggt y Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456ac tgt gcg ggt gtc gat aag gtt ccg ttc cct gag cgc atc aag a Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478tt gag gaa aac cac gag aac atc atg gct tgc gct aag tct cca e Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49tg gag aac act tgg tgg gct gag caa gat tct ccg ttc tgc ttc ctt u Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55ttc tgc ttt gag tac gct ggg gta cag cac cac ggc ctg agc tat a Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 aac tgc tcc ctt ccg ctg gcg ttt gac ggg tct tgc tct ggc atc cag n Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534tc tcc gcg atg ctc cga gat gag gta ggt ggt cgc gcg gtt aac s Phe Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556tt cct agt gaa acc gtt cag gac atc tac ggg att gtt gct aag u Leu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57aa gtc aac gag att cta caa gca gac gca atc aat ggg acc gat aac s Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589ta gtt acc gtg acc gat gag aac act ggt gaa atc tct gag aaa u Val Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6gtc aag ctg ggc act aag gca ctg gct ggt caa tgg ctg gct tac ggt l Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662ct cgc agt gtg act aag cgt tca gtc atg acg ctg gct tac ggg l Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634aa gag ttc ggc ttc cgt caa caa gtg ctg gaa gat acc att cag r Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ca gct att gat tcc ggc aag ggt ctg atg ttc act cag ccg aat cag 2 Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667ct gga tac atg gct aag ctg att tgg gaa tct gtg agc gtg acg 2 Ala Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68tg gta gct gcg gtt gaa gca atg aac tgg ctt aag tct gct gct aag 2 Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69ctg gct gct gag gtc aaa gat aag aag act gga gag att ctt cgc 2 Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77aag cgt tgc gct gtg cat tgg gta act cct gat ggt ttc cct gtg tgg 22Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ag gaa tac aag aag cct att cag acg cgc ttg aac ctg atg ttc ctc 2256 Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ag ttc cgc tta cag cct acc att aac acc aac aaa gat agc gag 23Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76tt gat gca cac aaa cag gag tct ggt atc gct cct aac ttt gta cac 2352 Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778aa gac ggt agc cac ctt cgt aag act gta gtg tgg gca cac gag 24Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79tac gga atc gaa tct ttt gca ctg aat cac gac tcc ttc ggt acc 2448 Lys Tyr Gly Ile Glu Ser Phe Ala Leu Asn His Asp Ser Phe Gly Thr 88ccg gct gac gct gcg aac ctg ttc aaa gca gtg cgc gaa act atg 2496 Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823ac aca tat gag tct tgt gat gta ctg gct gat ttc tac gac cag 2544 Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84tc gct gac cag ttg cac gag tct caa ttg gac aaa atg cca gca ctt 2592 Phe Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856ct aaa ggt aac ttg aac ctc cgt gac atc tta gag tcg gac ttc 264la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878tc gcg taa 2652 Ala Phe Ala PRT Artificial sequence Description of the artificial sequence mutated sequences of bacteriophage T7 RNA polymerase Asn Thr Ile Asn Ile Ala Lys Asn Asp Phe Ser Asp Ile Glu Leu Ala Ile Pro Phe Asn Thr Leu Ala Asp His Tyr Gly Glu Arg Leu 2 Ala Arg Glu Gln Leu Ala Leu Glu His Glu Ser Tyr Glu Met Gly Glu 35 4a Arg Phe Arg Lys Met Phe Glu Arg Gln Leu Lys Ala Gly Glu Val 5 Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys 65 7 Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg 85 9y Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu Val Ala Tyr Ile Thr Val Lys Thr Thr Leu Ala Cys Leu Thr Ser Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala Glu Asn Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp 2Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr 222et Val Ser Leu His Arg Gln Asn Ala Gly Val Val Gly Gln Asp 225 234lu Thr Ile Glu Leu Ala Pro Glu Tyr Ala Glu Ala Ile Ala Thr 245 25rg Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val 267ro Pro Lys Pro Trp Thr Gly Ile Thr Gly Gly Gly Tyr Trp Ala 275 28sn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala 29Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile 33Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala 325 33al Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile 345la Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp 355 36et Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val 378rg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe 385 39Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe 44Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Ser Met Phe 423ro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala Lys 435 44ly Lys Pro Ile Gly Lys Glu Gly Tyr Tyr Trp Leu Lys Ile His Gly 456sn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile Lys 465 478le Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro 485 49eu Glu Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe Leu 55Phe Cys Phe Glu Tyr Ala Gly Val Gln His His Gly Leu Ser Tyr 5525 Asn Cys Ser Leu Pro Leu Ala Phe Asp Gly Ser Cys Ser Gly Ile Gln 534he Ser Ala Met Leu Arg Asp Glu Val Gly Gly Arg Ala Val Asn 545 556eu Pro Ser Glu Thr Val Gln Asp Ile Tyr Gly Ile Val Ala Lys 565 57ys Val Asn Glu Ile Leu Gln Ala Asp Ala Ile Asn Gly Thr Asp Asn 589al Val Thr Val Thr Asp Glu Asn Thr Gly Glu Ile Ser Glu Lys 595 6Val Lys Leu Gly Thr Lys Ala Leu Ala Gly Gln Trp Leu Ala Tyr Gly 662hr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr Gly 625 634ys Glu Phe Gly Phe Arg Gln Gln Val Leu Glu Asp Thr Ile Gln 645 65ro Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn Gln 667la Gly Tyr Met Ala Lys Leu Ile Trp Glu Ser Val Ser Val Thr 675 68al Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala Lys 69Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Gly Glu Ile Leu Arg 77Lys Arg Cys Ala Val His Trp Val Thr Pro Asp Gly Phe Pro Val Trp 725 73ln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu 745ln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu 755 76le Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His 778ln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu 785 79Tyr Gly Ile Glu Ser Phe Ala Leu Asn His Asp Ser Phe Gly Thr 88Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met 823sp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln 835 84he Ala Asp Gln Leu His Glu Ser Gln Leu Asp Lys Met Pro Ala Leu 856la Lys Gly Asn Leu Asn Leu Arg Asp Ile Leu Glu Ser Asp Phe 865 878he Ala

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United States Patent **7,323,303**
Wong , et al. **January 29, 2008**

Modified .beta.-lactamases and uses thereof

Abstract

A tool that can screen bacteria for .beta.-lactamases against a panel of various antibiotics is desirable. A biosensor incorporating an indicator molecule into .beta.-lactamases may achieve this purpose, but it requires that the attached indicator molecule must not impair the binding affinity of the protein to a great extent to provide a higher sensitivity. A modified .beta.-lactamases with a residue on the .OMEGA.-loop or outside the .OMEGA.-loop but close to the active site of .beta.-lactamase being replaced by a reactive residue is developed in this invention.

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Claims

The invention claimed is:

1. A method for detecting .beta.-lactam antibiotics or .beta.-lactamase inhibitors in a sample, comprising the steps of: (1) providing a modified .beta.-lactamase for binding with said .beta.-lactam antibiotics or .beta.-lactamase inhibitors, wherein the modified .beta.-lactamase is a ***wild-type*** .beta.-lactamase having a residue at a position that corresponds to a position in the amino acid sequence of the Bacillus cereus .beta.-lactamase I selected from the group consisting of Ser70, Lys73, Asp104, Ser130, Asn163, Arg164, Phe165, Thr167, Glu168, Leu169, Asn170, Glu171, Ala172, lle173, Pro174, Gly175, Asp176, lle177, Arg178, and Lys234 replaced by an amino acid selected from the group consisting of an amino acid containing a free thiol group, an amino acid containing a free carboxylic acid group, and an amino acid containing a free amine group, and the replacing amino acid is covalently bonded to a fluorescent indicator for generating a change of fluorescence upon binding

.beta.-lactam antibiotics or .beta.-lactamase inhibitors, (2) exposing the sample to said modified .beta.-lactamase comprising a fluorescent indicator molecule, and (3) comparing the fluorescence emitted by the modified .beta.-lactamase bound with said .beta.-lactam antibiotics or .beta.-lactamase inhibitors, with the fluorescence emitted by the .beta.-lactamase from a control sample having no .beta.-lactam antibiotics or .beta.-lactamase inhibitor, whereby the presence of .beta.-lactam antibiotics or .beta.-lactamase inhibitors in a sample is detected.

2. The method as claimed in claim 1, wherein the replacing amino acid is cysteine.

3. The method as claimed in claim 1, wherein the indicator molecule is a fluorophore.

Description

FIELD OF THE INVENTION

This invention relates to modified .beta.-lactamases that can be used in the detection of .beta.-lactam antibiotics and .beta.-lactamase inhibitors, and/or in screening bacteria for .beta.-lactamases against a panel of .beta.-lactam antibiotics.

BACKGROUND OF THE INVENTION

.beta.-Lactam antibiotics (e.g. penicillins and cephalosporins) are an important class of antibacterial agents widely used in clinical therapies and as health promoting agents in animal feedstuffs. The clinical function of .beta.-lactam antibiotics relies on their inhibitory effect on the activity of penicillin-binding proteins which are responsible for synthesizing bacterial cell wall. However, the clinical importance of .beta.-lactam antibiotics has been challenged by the emergence of .beta.-lactamases which are capable of inactivating .beta.-lactam antibiotics by hydrolyzing the .beta.-lactam ring to carboxylic acid. To respond to this clinical problem, the pharmaceutical industry has produced a wide range of .beta.-lactam antibiotics (which have stronger resistance toward the hydrolyzing action of .beta.-lactamases) and new .beta.-lactamase inhibitors (which can irreversibly block the enzyme's active site via covalent modification). In order to search for a potent antibiotic from a large pool of drug candidates rapidly, a convenient tool that can screen bacteria for .beta.-lactamases against a panel of various antibiotics is desirable. Moreover, a sensing tool that can detect .beta.-lactamase inhibitors and .beta.-lactam antibiotics can also be useful. Such a tool can be used in the discovery of .beta.-lactam antibiotics and new .beta.-lactamase inhibitors, and utilized in routine measurement of antibiotic residues in liquid and food samples (e.g. milk).

OBJECTS OF THE INVENTION

Therefore, it is an object of this invention to resolve at least one or more of the problems as set forth in the prior art. As a minimum, it is an object of this invention to provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, this invention provides .beta.-lactamases with a non-reactive residue replaced by a reactive residue.

Preferably the reactive residue is selected from the group consisting of amino acids containing a free alcohol group, amino acids containing a free carboxylic acid group, or amino acids containing a free amine group. More preferably, the reactive residue is cysteine.

Preferably, the non-reactive residue is on the .OMEGA.-loop of said .beta.-lactamase.

Preferably, the reactive residue is further reacted with an indicator molecule to generate a signal to detect .beta.-lactam antibiotics or .beta.-lactamase inhibitors. More preferably, the indicator molecule is a fluorophore.

Optionally, the non-reactive residue is Glu-166 residue.

Preferably, the .beta.-lactamase is a mutant. More preferably, the .beta.-lactamase is a singly mutated mutant. Alternatively, the .beta.-lactamase is a multiply mutated mutant. For example, in one specific embodiment, the .beta.-lactamase is a E166C mutant.

A second aspect of this invention provides a method for detecting .beta.-lactam antibiotics or .beta.-lactamase inhibitors in a sample, including the steps of: exposing the sample to a .beta.-lactamase with a non-reactive residue on the .OMEGA.-loop replaced by a reactive residue, for binding said .beta.-lactamase with said .beta.-lactam antibiotics or .beta.-lactamase inhibitors; detecting a signal emitted by the .beta.-lactamase bound with said .beta.-lactam antibiotics or .beta.-lactamase inhibitors.

Preferably, the method of this invention further includes the step of comparing the signal emitted by the .beta.-lactamase bound with said .beta.-lactam antibiotics or .beta.-lactamase inhibitors, with a signal emitted by the .beta.-lactamase from a control sample having no .beta.-lactam antibiotics and .beta.-lactamase inhibitors.

Yet another aspect of this invention provides an apparatus for detecting .beta.-lactam antibiotics or .beta.-lactamase inhibitors in a sample incorporating a .beta.-lactamase with a non-reactive residue on the .OMEGA.-loop replaced by a reactive residue.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the present invention will now be explained by way of example and with reference to the accompany drawings in which:

FIG. 1 shows the tertiary structure of .beta.-lactamase I;

FIG. 2 shows the ESI mass spectrum of (A) E166C and (B) E166Cf mutants;

FIG. 3 shows the CD spectra of the wild-type .beta.-lactamase I, E166C and E166Cf mutants at the same concentration (6.0.times.10.sup.-6 M) in 50 mM phosphate buffer (pH 7.0);

FIG. 4 shows the Fluorescence spectra of the E166Cf enzyme (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M penicillin G (a), 1.0.times.10.sup.-5 M penicillin G (b), 1.0.times.10.sup.-6 M penicillin G (c), 1.0.times.10.sup.-7 M penicillin G (d), 1.0.times.10.sup.-8 M penicillin G (e) and 0 M penicillin G (f). The E166Cf enzymes were incubated with various concentrations of penicillin G for 130 s at room temperature before

measurement. Excitation wavelength: 494 nm;

FIG. 5 shows the Time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M penicillin G (a), 1.0.times.10.sup.-5 M penicillin G (b), 1.0.times.10.sup.-6 M penicillin G (c), 1.0.times.10.sup.-7 M penicillin G (d) and 1.0.times.10.sup.-8 M penicillin G (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (penicillin G, M);

FIG. 6 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M penicillin V (a), 1.0.times.10.sup.-5 M penicillin V (b), 1.0.times.10.sup.-6 M penicillin V (c), 1.0.times.10.sup.-7 M penicillin V (d) and 1.0.times.10.sup.-8 M penicillin V (e). Excitation wavelength: 494 mn. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (penicillin V, M);

FIG. 7 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M ampicillin (a), 1.0.times.10.sup.-5 M ampicillin (b), 1.0.times.10.sup.-6 M ampicillin (c), 1.0.times.10.sup.-7 M ampicillin (d) and 1.0.times.10.sup.-8 M ampicillin (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (ampicillin, M);

FIG. 8 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M cefuroxime (a), 1.0.times.10.sup.-5 M cefuroxime (b), 1.0.times.10.sup.-6 M cefuroxime (c), 1.0.times.10.sup.-7 M cefuroxime (d) and 1.0.times.10.sup.-8 M cefuroxime (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (cefuroxime, M);

FIG. 9 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M cefoxitin (a), 1.0.times.10.sup.-5 M cefoxitin (b), 1.0.times.10.sup.-6 M cefoxitin (c) and 1.0.times.10.sup.-7 M cefoxitin (d). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (cefoxitin, M);

FIG. 10 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M moxalactam (a), 1.0.times.10.sup.-5 M moxalactam (b), 1.0.times.10.sup.-6 M moxalactam (c), 1.0.times.10.sup.-7 M moxalactam (d) and 1.0.times.10.sup.-8 M moxalactam (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (moxalactam, M);

FIG. 11 shows the time-resolved fluorescence measurements of free fluorescein (1.2.times.10.sup.-7 M) at 512 nm in 50 mM phosphate buffer (pH 7.0) in the presence of unlabeled E166C enzyme (1.2.times.10.sup.-7 M) with (A) 1.0.times.10.sup.-5 M penicillin G, (B) 1.0.times.10.sup.-5 M penicillin V, (C) 1.0.times.10.sup.-5 M ampicillin, (D) 1.0.times.10.sup.-5 M cefuroxime, (E) 1.0.times.10.sup.-5 M cefoxitin and (F) 1.0.times.10.sup.-5 M moxalactam as substrates. Excitation wavelength: 494 nm;

FIG. 12 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-6 M penicillin G (a) and 1.0.times.10.sup.-7 M penicillin G (b). Excitation wavelength: 494 nm;

FIG. 13 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M penicillin G (a) and 1.0.times.10.sup.-5 M penicillin G (b). Excitation wavelength: 494 nm;

FIG. 14 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0.times.10.sup.-4 M cefoxitin (a) and 1.0.times.10.sup.-6 M cefoxitin (b). Excitation wavelength: 494 nm;

FIG. 15 shows the circular dichroism signals (258 nm) of cefuroxime in 50 mM phosphate buffer (pH 7.0) as a function of time in the presence of E166Cf enzyme (1.2.times.10.sup.-7 M);

FIG. 16 shows the circular dichroism signals (264 nm) of cefoxitin in 50 mM phosphate buffer (pH 7.0) as a function of time in the presence of E166Cf enzyme (1.2.times.10.sup.-7 M);

FIG. 17 shows the circular dichroism signals (265 nm) of moxalactam in 50 mM phosphate buffer (pH 7.0) as a function of time in the presence of E166Cf enzyme (1.2.times.10.sup.-7 M);

FIG. 18 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) with 1.0.times.10.sup.-4 M sulbactam as substrate. Excitation wavelength: 494 nm;

FIG. 19 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) with 1.0.times.10.sup.-3 M clavulanate as substrate. Excitation wavelength: 494 nm;

FIG. 20 shows the (A) A 96-well microtiter plate (Coming Costar) and (B) the schematic diagram of the set-up used in the detection of .beta.-lactam antibiotics in milk and in drug screening experiments;

FIG. 21 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 520 nm in untreated milk in the presence of 1.0.times.10.sup.-4 M penicillin G (a), 1.0.times.10.sup.-5 M penicillin G (b), 1.0.times.10.sup.-6 M penicillin G (c), 1.0.times.10.sup.-7 M penicillin G (d) and in the absence of penicillin G (e). Excitation wavelength: 485 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (penicillin G, M);

FIG. 22 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 520 nm in untreated milk in the presence of 1.0.times.10.sup.-4 M ampicillin (a), 1.0.times.10.sup.-5 M ampicillin (b), 1.0.times.10.sup.-6 M ampicillin (c), 1.0.times.10.sup.-7 M ampicillin (d) and in the absence of ampicillin (e). Excitation wavelength: 485 nm. The inset shows the plot of the change in fluorescence intensity (at the peak maxima) versus log (ampicillin, M);

FIG. 23 shows the time-resolved fluorescence measurements of the El 66Cf enzyme (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) with (A) penicillin G (1.0.times.10.sup.-4 M), (B) penicillin V (1.0.times.10.sup.-4 M), (C) ampicillin (1.0.times.10.sup.-4 M), (D) cefuroxime (1.0.times.10.sup.-4 M), (E) cefoxitin (1.0.times.10.sup.-4 M) and (F) moxalactam (1.0.times.10.sup.-4 M) as substrates in the absence of .beta.-lactamase II (a) and in the presence of .beta.-lactamase II (b). Excitation wavelength: 485 nm;

FIG. 24 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) with (A) penicillin G (1.0.times.10.sup.-4 M), (B) penicillin V (1.0.times.10.sup.-4 M), (C) ampicillin (1.0.times.10.sup.-4 M), (D) cefuroxime (1.0.times.10.sup.-4 M), (E) cefoxitin (1.0.times.10.sup.-4 M) and (F) moxalactam (1.0.times.10.sup.-4 M) as substrates in the absence of penPC .beta.-lactamase (a) and in the presence of penPC .beta.-lactamase (b). Excitation wavelength: 485 nm;

FIG. 25 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) with (A) penicillin G (1.0.times.10.sup.-4 M), (B) penicillin V (1.0.times.10.sup.-4 M), (C) ampicillin (1.0.times.10.sup.-4 M), (D) cefuroxime (1.0.times.10.sup.-4 M), (E) cefoxitin (1.0.times.10.sup.-4 M) and (F) moxalactam (1.0.times.10.sup.-4 M) as substrates in the absence of penP .beta.-lactamase (a) and in the presence of penP .beta.-lactamase (b). Excitation wavelength: 485 nm; and

FIG. 26 shows the time-resolved fluorescence measurements of the E166Cf enzyme (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) with (A) penicillin G (1.0.times.10.sup.-4 M), (B) penicillin V (1.0.times.10.sup.-4 M), (C) ampicillin (1.0.times.10.sup.-4 M), (D) cefuroxime (1.0.times.10.sup.-4 M), (E) cefoxitin (1.0.times.10.sup.-4 M) and (F) moxalactam (1.0.times.10.sup.-4 M) as substrates in the absence of TEM-1 .beta.-lactamase (a) and in the presence of TEM-1 .beta.-lactamase (b). Excitation wavelength: 485 nm.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention is now described by way of example with reference to the figures in the following paragraphs.

Objects, features, and aspects of the present invention are disclosed in or are obvious from the following description. It is to be understood by one of ordinary skill in the art that the present discussion is a description of exemplary embodiments only, and is not intended as limiting the broader aspects of the present invention, which broader aspects are embodied in the exemplary constructions.

One way to satisfy the objects mentioned above is to develop a biosensor by incorporating a reporter group or indicator molecule (e.g. luminescent probes) into a biomolecule (e.g. .beta.-lactamase or any penicillin binding proteins) such that the reporter group can transform the substrate or inhibitor-binding event into a measurable signal. This can be achieved by incorporating a reactive residue, for example thiol-containing cysteine, into a protein by site-directed mutagenesis, and then labeling the cysteine residue with an indicator molecule, for example a thiol-reactive reporter group. Such biosensors offer a number of advantages, including high sensitivity, high specificity, simplicity and low cost. However, this approach requires that the attached indicator molecule must not impair the binding affinity of the protein to a great extent to provide a higher sensitivity.

The structure and amino acid sequence of .beta.-lactamase can be found in the following References 1 to 5, respectively: 1. Madgwick, P. J. and Waley, S. G. .beta.-lactamase I from Bacillus cereus. Structure and site-directed mutagenesis (1987) Biochem. J. 248, 657-662. 2. Aschaffenburg, R., Phillips, D. C, Sutton, B. J., Baldwin, G., Kiener, P. A., Waley, S. G. Preliminary crystallographic data for .beta.-lactamase I from Bacillus cereus 569. (1978) J. Mol. Biol. 120, 447-449. 3. Samraoui, B., Sutton, B. J., Todd, R. J., Artymiuk, P. J., Waley, S. G., Phillips, D. C. Tertiary structural similarity between a class A .beta.-lactamase and a penicillin-sensitive D-anlanyl carboxypeptidase-transpeptidase. (1986) Nature 320, 378-380. 4. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., Frere, J. M., .beta.-lactamase Bacillus licheniformis 749/C at 2 .ANG. resolution. (1990) Proteins 7, 156-171. 5. Ambler, R. P.; Coulson, A. F. W, Frere, J.-M.; Ghuysen, J.-M.; Joris, B.; Forsman, M.; Levesque, R. C.; Tiraby, G.; Waley, S. G. A standard numbering scheme for the Class A .beta.-lactamase, (1991), Biochem. J. 276, 269-272.

In this invention, a biosensor for .beta.-lactam antibiotics and .beta.-lactamase inhibitors is provided by attaching an indicator molecule close to the active site of a .beta.-lactamase. In a specific embodiment of this invention, the indicator molecule is an environmentally sensitive fluorescein molecule, and the .beta.-lactamase is a mutant. The mutant (E166C) was constructed in which a residue on the .OMEGA.-loop of the wild-type .beta.-lactamase I was replaced by a reactive residue by site-directed mutagenesis. The reactive residue can be any residue having a free function group to react with an indicator molecule. For example, amino acids containing a free alcohol group such as serine, threonine and tyrosine may be used. These amino acids can be derivatised with salicylaldehyde, which can then be coupled to fluorescent reagents such as 1,2-diamino-4,5-dimethoxybenzene. Amino acids containing a free carboxylic acid group such as glutamic acid and aspartic acid may also be used, which can be coupled to carbodiimides which then react with a fluorescent reagent, or to carbodiimides which contain a fluorophore. A further option is to use amino acids containing a free amine group such as lysine, which can be coupled to isothiocyanantes or succinimidyl esters.

In one specific embodiment, the replaced residue is the Glu-166 residue (according to the ABL number system, Reference 5), and the reactive residue is a cysteine residue. The mutant was then labeled with an indicator molecule, in one specific embodiment a thiol-reactive fluorescein-5-maleimide, at its reactive residue, the cysteine residue, to form E166Cf, a specific modified .beta.-lactamase of this invention.

It should be emphasized that this invention shall not be limited by the E166Cf constructed. The reside to be replaced on the .OMEGA.-loop of the wild-type .beta.-lactamase can be at various positions including Asn163, Arg164, Phe165, Glu166, Thr167, Glu168, Leu169, Asn170, Glu171, Ala172, Ile173, Pro174, Gly175, Asp176, Ile177, Arg178, though preferably to be the Glu-166 residue. Further, residues outside the .OMEGA.-loop but close to the active site can also be used, e.g. Ser70, Lys73, Asp104, Ser130, and Lys234. The .beta.-lactamase can be wild type .beta.-lactamase or its mutant, whether it is a singly or multiply mutated mutant, in which a single mutated mutant contains only one amino acid substitution while a multiply mutated mutant contains more than one amino acid substitutions. Further, the indicator molecule can be any other molecule capable of emitting a detectable signal other than fluorescent signal. However, fluorophore capable of emitting fluorescent signal as fluorescent signal may create less influence to the structure of the enzyme.

Referring to the specific E166Cf, as the wild-type .beta.-lactamase I has no cysteine, incorporation of a cysteine residue into the protein allows the fluorophore to be specifically attached at the desired site. The Glu-166 residue was chosen as the labeling site not only because of its closeness to the enzyme's active site, but also the flexibility of the .OMEGA.-loop which allows the attached fluorescein

molecule to move when a substrate enters the active site. Thus, the E166Cf mutant can serve as a reagentless tool to detect .beta.-lactam antibiotics with high sensitivity and specificity. Moreover, substrate competition between the labeled enzyme (E166Cf) and bacterial .beta.-lactamase will lead to a change in antibiotic concentration (e.g. decrease in antibiotic concentration due to the hydrolytic action of bacterial .beta.-lactamase) and hence generating different fluorescence signals, thus allowing the labeled enzyme to be used conveniently in drug screening, selecting the most appropriate .beta.-lactam antibiotic for patients with bacterial infection.

The synthesis of E166Cf and its use in detecting .beta.-lactam antibiotics and .beta.-lactamase inhibitors, and/or in screening bacteria for .beta.-lactamases against a panel of .beta.-lactam antibiotics will now be described in details. However, as there are various options in designing the modified .beta.-lactamase as mentioned above, suitable modifications may be necessary to the below methods, which are readily available to a person skilled in the art.

Expression and purification of wild-type .beta.-lactamase I and E166C mutant were performed as described previously (Reference 6: Leung, Y. C. Robinson, C. V., Alpin, R. T., Waley, S. G., Site-directed synthesis of .beta.-lactamase I: role of Glu-166 (1994) Biochem. J. 299, 671-678). with slight modifications. Both wild-type .beta.-lactamase I and E166C mutant were expressed in B. subtilis 1A304 (.phi.105MU331). A bacterial strain was streaked on an agar plate containing 5 .mu.g/ml chloramphenicol, and the plate was incubated at 37.degree. C. for 24 h. A single bacterial colony from the agar plate was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) containing 5 .mu.g/ml chloramphenicol, which was then incubated at 37.degree. C. with shaking at 300 rpm overnight. About 2 ml of overnight inoculum was added to each of four conical flasks containing 100 ml of sterilized BHY medium. The inoculated media were then incubated at 37.degree. C. with shaking at 300 rpm. When the OD.sub.600 reached 3.5-4.0, the bacterial cultures were heated in a water bath at 51.degree. C. for 5 min and then incubated at 37.degree. C. with shaking at 300 rpm for a further 6 h. The supernatant of the bacterial cultures was collected by centrifugation (9000 rpm) at 4.degree. C. for 25 min, and adjusted to pH 7.0 with conc. HCl. The .beta.-lactamases were extracted by mixing the supernatant with 40 g of celite 545 for 30 min in an ice bath. After discarding the supernatant, the celite was washed three to four times with 300 ml of deionized water. The .beta.-lactamases were collected by mixing the celite three times with 100 ml of protein elution buffer (100 mM Tris-HCl, 2 M NaCl and 100 mM tri-sodium citrate, pH 7.0). The protein solution was filtered by suction and then concentrated to 10 ml at 4.degree. C. using a concentrator (Amicon) equipped with a piece of YM-1 membrane (MWCO=1,000). The concentrated protein solution was exchanged with 20 mM NH.sub.4HCO.sub.3 and then freeze-dried. The enzyme powder was stored at -80.degree. C. About 15 mg of wild-type .beta.-lactamase I and 20 mg of E166C mutant were obtained by the above procedures.

Protein Labeling

About 2.5 mg of E166C mutant was dissolved in 4 ml of 6 M guanidine hydrochloride. The protein solution was incubated at room temperature for 30 min to unfold the mutant. A ten-fold molar excess of fluorescein-5-maleimide (purchased from Molecular Probes) dissolved in dimethylsulfoxide was added to the protein solution, and the pH of the mixture was adjusted to 7.5 with 0.2 M NaOH. The mixture was stirred at room temperature for 2 h in dark, and then dialyzed with a dialysis tubing (MWCO=12,000) against 1 L of 20 mM NH.sub.4HCO.sub.3 (pH 7.0) at 4.degree. C. for about 3 days to remove the free dyes. Buffer exchanges were carried out regularly during dialysis. After dialysis, the labeled mutant (E166Cf) was freeze-dried and stored at -80.degree. C.

Characterization of the E166Cf Enzyme

The labeling of the E166C mutant with fluorescein-5-maleimide was monitored by electrospray ionization mass spectrometry (ESI/MS). The mass spectrometric measurements were performed on a VG Platform mass spectrometer (Micromass) equipped with an electrospray interface. Protein samples (20 .mu.l) were injected into the electrospray source via a loop injector as solution in H.sub.2O/CH.sub.3CN (1:1) containing formic acid (0.2%, v/v). The mass difference between the E166C and E166Cf mutants (FIG. 2) is consistent with the molecular mass of fluorescein-5-maleimide (MW=427) plus the mass of a sodium ion. The mass spectrum of the E166Cf enzyme indicate that almost all the E166C mutants were labeled with fluorescein molecules.

The secondary structure of the wild-type .beta.-lactamase I, E166C and E166Cf were investigated by circular dichroism (CD) spectropolarimetry. The CD measurements were performed on a Jasco J810 Spectropolarimeter (Jasco Co.). The CD spectra of the wild-type .beta.-lactamase I, E166C and E166Cf mutants at the same concentration (6.0.times.10.sup.-6 M) in 50 mM phosphate buffer (pH 7.0) on the far-UV region are shown in FIG. 3. The CD signals exhibit no significant difference, indicating that the secondary structure of the E166Cf enzyme is conserved after labeling with fluorescein.

The hydrolytic activities of the wild-type .beta.-lactamase I, E166C and E166Cf mutants towards penicillin G, penicillin V and ampicillin (Sigma) were monitored by the spectrophotometric method. The spectrophotometric measurements were performed on a Perkin Elmer Lambda Bio20 UV/Vis spectrometer. Substrate hydrolysis was monitored at a fixed wavelength: 235 nm for ampicillin, 232 nm for penicillin G and penicillin V. The initial rate of substrate hydrolysis occurring within 5 min was determined in duplicate at each of 6 different substrate concentrations in 50 mM potassium phosphate buffer (pH 7.0) at 20.degree. C. The initial rates determined were then treated as described in Reference 5 to calculate the Michaelis constants (K.sub.m) and turnover numbers (k.sub.cat) using non-linear regression analysis (equation 1):

##EQU00001## where v is the initial rate of substrate hydrolysis, V.sub.max the maximum rate of reaction, [S] the initial substrate concentration, K.sub.m the Michaelis constant and k.sub.cat=V.sub.max/[Enzyme].

The measured steady-state kinetic parameters for hydrolysis of penicillin G, penicillin V and ampicillin by the wild-type .beta.-lactamase I, E166C and E166Cf mutants are summarized in Table 1. The results indicate that the hydrolytic activity of the labeled enzyme is conserved after labeling with fluorescein.

TABLE-US-00001 TABLE 1 K.sub.m (.mu.M) Wild-type E166C E166Cf Penicillin G 48 .+-. 3 72 .+-. 3 213 .+-. 11 Penicillin V 52 .+-. 4 71 .+-. 6 117 .+-. 10 Ampicillin 142 .+-. 8 306 .+-. 30 262 .+-. 31 k.sub.cat (s.sup.-1) Wild-type E166C E166Cf Penicillin G 2612 .+-. 320 2.07 .+-. 0.02 5.28 .+-. 0.09 Penicillin V 2109 .+-. 4 1.53 .+-. 0.03 2.97 .+-. 0.06 Ampicillin 5213 .+-. 275 4.1 .+-. 0.1 6.2 .+-. 0.2 k.sub.cat/K.sub.m (.mu.M.sup.-1s.sup.-1) Wild-type E166C E166Cf Penicillin G 54 .+-. 10 0.029 .+-. 0.002 0.025 .+-. 0.002 Penicillin V 41 .+-. 4 0.021 .+-. 0.002 0.025 .+-. 0.003 Ampicillin 37 .+-. 4 0.013 .+-. 0.002 0.023 .+-. 0.004

Fluorescence Measurements of the E166Cf Enzyme in the Presence of Penicillins and Cephalosporins

Fluorescence measurements of the E166Cf enzyme in the presence of penicillin G, penicillin V,

ampicillin, cefuroxime, cefoxitin and moxalactam were performed on a Perkin Elmer LS50B spectrofluorometer. For time-resolved fluorescence measurement, excitation and emission wavelengths were set at 494 and 515 nm respectively. Both excitation and emission slit widths were set at 5 nm. All fluorescence measurements were performed at room temperature.

With penicillin G as substrate, the fluorescence signal of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) increases as a function of the antibiotic concentration (FIG. 4). The time-resolved fluorescence signals of the E166Cf enzyme at 515 nm were also measured with various concentrations of penicillin G (FIG. 5). At low antibiotic concentration (1.0.times.10.sup.-7 and 1.0.times.10.sup.-6 M), the fluorescence intensity of the E166Cf enzyme increases gradually and then declines. At high antibiotic concentration (1.0.times.10.sup.-5 and 1.0.times.10.sup.-4 M), the fluorescence signal increases instantaneously and levels off to a plateau. The fluorescence signal stays at the plateau for a certain length of time and then declines afterwards. Similar results were obtained with penicillin V and ampicillin (FIGS. 6 and 7).

With cefuroxime, cefoxitin and moxalactam as substrates, the fluorescence intensities of the E166Cf enzyme (1.2.times.10.sup.-7 M) at 515 nm in 50 mM phosphate buffer (pH 7.0) also increase as a function of antibiotic concentration (FIGS. 8 to 10). The E166Cf enzyme exhibited increasing fluorescence signals with antibiotic concentration, but no subsequent decline in intensity was observed even after one hour.

As the fluorescence intensity of fluorescein is known to change with pH, investigations were also made to see if the observed fluorescence changes were a result of the change in pH upon hydrolysis of the antibiotics by the labeled enzyme. First of all, the fluorescence intensity of fluorescein is known to decrease when the pH is lowered. So the observed increase in fluorescence intensity in the presence of antibiotics is not consistent with the generation of carboxylic acids upon hydrolysis by the enzyme. After the completion of hydrolysis, the overall change in pH of the bulk phosphate buffer solution as monitored by a pH electrode was less than 0.5 pH unit for penicillin G (1.0.times.10.sup.-5 M). When the fluorescence intensity of free fluorescein (1.2.times.10.sup.-7 M) in 50 mM phosphate buffer (pH 7.0) was measured with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0.times.10.sup.-5 M) in the presence of unlabeled E166C enzyme (1.2.times.10.sup.-7 M), it remains almost unchanged throughout the whole experiment (FIG. 11) while the E166C enzyme was hydrolyzing the penicillin antibiotics (data not shown). Moreover, cephalosporins, which are known to be poor substrates for the E166C enzyme, also enhance the fluorescence signals. So the possibility that the increase in fluorescence signal of the E166Cf enzyme is due to the change in pH of the bulk solution can be eliminated. Furthermore, the possibility that the observed change in fluorescence signal is due to a change in local pH at the enzyme's active site can also be eliminated because it is unlikely that such a change in pH at the active site takes hundreds to thousands of seconds as shown in the time-resolved fluorescence measurements.

Without any binding theory, the observed fluorescence signals is believed to be attributed to the conformational change in the active site upon substrate binding. Upon binding to antibiotics, the fluorescein label attached to the flexible .OMEGA.-loop may move away from the catalytic pocket such that it is well separated from the quenchers (amino acids) in the active site. As a result, the fluorescence of the fluorescein molecule is restored.

The time-resolved fluorescence signals of the E166Cf enzyme obtained in the presence of penicillin and cephalosporin antibiotics at various concentrations can be rationalized by the following three-step model:

##STR00001## where E is the free .beta.-lactamase enzyme, S the .beta.-lactam substrate, ES a noncovalent enzyme-substrate complex, ES* an acyl-enzyme complex and P the carboxylic acid.

At low substrate concentration (1.0.times.10.sup.-7 and 1.0.times.10.sup.-6 M), the binding between the E166Cf enzyme (E) and penicillin substrate (S) would lead to the formation of the ES complex and hence the enhancement in the fluorescence signal. As hydrolysis of antibiotic proceeds, the majority of the penicillin substrates are converted to free carboxylic acids (P). As a result, most of the E166Cf enzymes return to their substrate-free conformation (E) and hence their original weak fluorescence signals were restored (FIG. 12). This may explain the slow decline in fluorescence signal with time after the peak.

At high substrate concentration (1.0.times.10.sup.-5 and 1.0.times.10.sup.-4 M), the penicillin substrates rapidly occupy the active sites to form the ES complexes, thus switching on the fluorescence of the fluorescein labels instantaneously. Because the antibiotic concentration is high, the continuous hydrolysis of antibiotics would maintain the E166Cf enzymes in the ES* state for a while, and hence the fluorescence signal is leveled off to a plateau (FIG. 13). This can be verified by the fact that the `plateau time` (8 and 22 min for 1.0.times.10.sup.-5 and 1.0.times.10.sup.-4 M penicillin G respectively) is consistent with the `substrate hydrolysis time` determined by spectrophotometric assays (7 and 20 min for 1.0.times.10.sup.-5 and 1.0.times.10.sup.-4 M penicillin G respectively). When most of the penicillin substrates have been hydrolyzed to carboxylic acids, the fluorescence signal of the labeled enzyme will decline subsequently.

The absence of a declining fluorescence signal for cefuroxime, cefoxitin and moxalactam can be ascribed to the poor hydrolytic activities of the E166Cf enzyme towards these antibiotics (very low k.sub.2 and/or k.sub.3) (FIG. 14). As a result, the cephalosporin substrates stay in the active sites. To verify this, the activities of the labeled enzyme with cefuroxime, cefoxitin and moxalactam as substrates were monitored by circular dichroism (CD) spectropolarimetry. The principle of this assay is that .beta.-lactam antibiotics, due to the asymmetric property of their fused ring systems, exhibit strong CD signals, but will become CD inactive when they are hydrolyzed by .beta.-lactamase. Therefore, by measuring the CD signals of cefuroxime, cefoxitin and moxalactam (1.0.times.10.sup.-4 M) at 258, 264 and 265 nm respectively as a function of time in the presence of E166Cf enzyme (1.2.times.10.sup.-7 M), the hydrolytic activities of the labeled enzyme towards these antibiotics can be monitored. As shown in FIGS. 15 to 17, no significant changes in the CD signals appear after incubating the E166Cf enzymes with the cephalosporin antibiotics for one hour. This indicates that cefuroxime, cefoxitin and moxalactam are resistant to the hydrolytic activity of the labeled enzyme.

Fluorescence Measurements of the E166Cf Enzyme in the Presence of .beta.-Lactamase Inhibitors

Time-resolved fluorescence measurements of the E166Cf enzyme in the presence of sulbactam and clavulanate were performed on a Perkin Elmer LS50B spectrofluorometer. The excitation and emission wavelengths were set at 494 and 515 nm respectively. Both excitation and emission slit widths were set at 5 nm. All fluorescence measurements were performed at room temperature.

The fluorescence signals of the labeled enzyme (1.2.times.10.sup.-7 M) obtained with sulbactam (1.0.times.10.sup.-4 M) and clavulanate (1.0.times.10.sup.-3 M) in 50 mM phosphate buffer (pH 7.0) are entirely different (FIGS. 18 and 19). For sulbactam, the fluorescence intensity increases rapidly at the initial stage and then declines slowly. For clavulanate, the addition of substrate causes an instantaneous increase in fluorescence signal which declines rapidly within 200 s and then levels off to

a plateau. These results indicate that the E166Cf enzyme can be used to detect .beta.-lactamase inhibitors.

Detection of .beta.-Lactam Antibiotics in Milk

Fluorescence measurements of the E166Cf enzyme in milk in the presence of penicillin G and ampicillin were performed on a FLUOstar microplate reader (BMG Labtechnologies) equipped with two sample injectors. Excitation and emission filters of 485 and 520 nm respectively were used. Milk (pasteurized and homogenized, Nestle Dairy Farm) was purchased at a local supermarket. The labeled enzyme was mixed with the milk sample in a 96-well microtiter plate (Corning Costar). The antibiotics were then added to the mixtures by the injectors. The experimental set-up is shown in FIG. 20.

FIGS. 21 and 22 show the results from the time-resolved fluorescence measurements of the labeled enzyme (1.2.times.10.sup.-7 M) in untreated milk in the presence of various concentrations of penicillin G and ampicillin respectively. The results indicate that the labeled enzyme is capable of detecting penicillin G and ampicillin down to 10.sup.-6 M. Therefore, the labeled enzyme may find its application in the routine measurement of antibiotics in liquid samples (e.g. milk).

Application of the E166Cf Enzyme in Screening Bacteria for .beta.-Lactamases against a Panel of .beta.-Lactam Antibiotics

The use of the E166Cf enzyme in screening bacterial .beta.-lactamases against various .beta.-lactam antibiotics was investigated. The principle of our method is that when both E166Cf and bacterial .beta.-lactamase are incubated with `.beta.-lactamase-unstable` antibiotics, the fluorescence of the E166Cf enzyme will be suppressed because of the greater catalytic efficiency of the bacterial enzyme. In contrast, when bacterial .beta.-lactamases are screened against `.beta.-lactamase-resistant` antibiotics, the fluorescence of the E166Cf enzyme will be enhanced because of the poor hydrolytic activity of the bacterial enzyme. Thus, any .beta.-lactam antibiotic that causes the labeled enzyme to increase its fluorescence intensity in the presence of bacterial .beta.-lactamases can be used in clinical treatments.

In this invention, B. cereus penPC .beta.-lactamase, B. licheniformis penP .beta.-lactamase, E. coli. TEM-1 .beta.-lactamase and B. cereus .beta.-lactamase II. were tested. B. cereus .beta.-lactamase II is classified as Class B .beta.-lactamase whereas the others are classified as Class A .beta.-lactamase. Thus, we are interested to investigate whether the labeled enzyme can be used in screening different classes of .beta.-lactamase against various .beta.-lactam antibiotics.

Preparation of Bacterial .beta.-lactamase

Bacterial .beta.-lactamases used in the drug screening experiments were B. cereus penPC .beta.-lactamase, B. licheniformis penP .beta.-lactamase, E. coli. TEM-1 .beta.-lactamase and B. cereus .beta.-lactamase II.

The .beta.-lactamase II, penPC .beta.-lactamase and penP .beta.-lactamase were expressed in B. subtilis 1A304 (.phi.105MU331). These enzymes were prepared according to the procedures described previously (Reference 7: Thomwell, S. J. East, A. K., Errington, J. An efficient expression and secretion system based on Bacillus subtilis phage phi 105 and its uses for the production for B. cereus .beta.-lactamase I. (1993) Gene 133, 47-53) with slight modifications. A bacterial strain was streaked

on an agar plate containing 5 .mu.g/ml chloramphenicol, and the plate was incubated at 37.degree. C. for 24 h. A single bacterial colony from the agar plate was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) which was then incubated at 37.degree. C. with shaking at 300 rpm overnight. About 7 ml of overnight inoculum was added to a baffled conical flask containing 100 ml of sterilized BHY medium. The inoculated medium was incubated at 37.degree. C. with shaking at 300 rpm. When the optical density of the bacterial culture at 600 nm reached 3.5 to 4.0, the bacterial culture was heated in a water bath at 51.degree. C. for 5 min. Afterwards, the bacterial culture was incubated at 37.degree. C. with shaking at 300 rpm for a further 6 h. The bacterial culture was then harvested and stored at -20.degree. C.

The TEM-1 .beta.-lactamase was expressed in E. coli. BL21(DE3). Preparation of the TEM-1 .beta.-lactamase was performed as follows. A bacterial strain was streaked on an agar plate containing 100 .mu.g/ml ampicillin, and the plate was incubated at 37.degree. C. for 24 h. A single bacterial colony from the plate was inoculated into 100 ml of sterilized LB broth (28 g/l) which was then incubated at 37.degree. C. with shaking at 280 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at -20.degree. C.

Preparation of B. subtilis 1A304 (.phi.105MU331) and E. coli. BL21(DE3) Cultures

Both B. subtilis 1A304 (.phi.105MU331) and E. coli. BL21(DE3) cultures (which produce no .beta.-lactamase) were prepared as negative controls for drug screening experiments. Preparations of B. subtilis 1A304 (.phi.105MU331) and E. coli. BL21(DE3) cultures were performed as follows.

For B. subtilis 1A304 (.phi.105MU331), a bacterial strain was streaked on an agar plate containing 5 .mu.g/ml chloramphenicol, and the plate was incubated at 37.degree. C. for 24 h. A single bacterial colony was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) which was then incubated at 37.degree. C. with shaking at 300 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at -20.degree. C. For E. coli. BL21(DE3), a bacterial strain was streaked on an agar plate containing 100 .mu.g/ml ampicillin, and the plate was incubated at 37.degree. C. for 24 h. A single bacterial colony was inoculated into 100 ml of sterilized LB broth (28 g/l) which was then incubated at 37.degree. C. with shaking at 280 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at -20.degree. C.

Drug Screening Experiments

Fluorescence measurements of the E166Cf enzyme in the presence of bacterial cultures and .beta.-lactam antibiotics were performed on a FLUOstar microplate reader (BMG Labtechnologies) equipped with two sample injectors. Excitation and emission filters of 485 and 520 nm respectively were used. For B. cereus penPC .beta.-lactamase, B. licheniformis penP .beta.-lactamase and B. cereus .beta.-lactamase II, the labeled enzymes were mixed with the bacterial cultures, and the mixtures were made up to 300 .mu.l with 50 mM phosphate buffer (pH 7.0) in a 96-well microtiter plate (Corning Costar). For the purpose of comparison, B. subtilis cultures, which produced no .beta.-lactamase, were set up as negative controls. For E. coli. TEM-1 .beta.-lactamase, the labeled enzymes were mixed with the bacterial cultures without dilution. E. coli. cultures, which produced no .beta.-lactamase, were set up as negative controls for comparison. The antibiotics were then added to the bacterial samples by the injectors. The experimental set-up is shown in FIG. 20.

The results from the time-resolved fluorescence measurements of the labeled enzyme (1.2.times.10.sup.-7 M) in the presence of various bacterial cultures and .beta.-lactam antibiotics

(1.0.times.10.sup.-4 M) are shown in FIGS. 23 to 26. Table 2 summarizes the results obtained from the time-resolved fluorescence measurements.

TABLE-US-00002 TABLE 2 .beta.-Lactamase B. cereus .beta.- B. cereus B. licheniformis E. coli lactamase II penPC penP TEM-1 Penicillin G - - - - Penicillin V - - - - Ampicillin - - - - Cefuroxime - + - + Cefoxitin + + + + Moxalactam + + + + - Decline in fluorescence signal (presence of beta-lactam hydrolysis) + No decline in fluorescence signal (absence of beta-lactam hydrolysis)

The results indicate that cefoxitin and moxalactam are resistant to the hydrolytic activities of the bacterial .beta.-lactamases, and therefore can be used in the clinical treatment if patients are infected with bacteria which produce such .beta.-lactamases.

While the preferred embodiment of the present invention has been described in detail by the examples, it is apparent that modifications and adaptations of the present invention will occur to those skilled in the art. Furthermore, the embodiments of the present invention shall not be interpreted to be restricted by the examples or figures only. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims. For instance, features illustrated or described as part of one embodiment can be used on another embodiment to yield a still further embodiment. Thus, it is intended that the present invention cover such modifications and variations as come within the scope of the claims and their equivalents.

* * * * *

United States Patent **7,332,174**
Green , et al. **February 19, 2008**

Mutant forms of cholera holotoxin as an adjuvant

Abstract

Mutant cholera holotoxins having single or double amino acid substitutions or insertions have reduced toxicity compared to the wild-type cholera holotoxin. The mutant cholera holotoxins are useful as adjuvants in antigenic compositions to enhance the immune response in a vertebrate host to a selected antigen from a pathogenic bacterium, virus, fungus, or parasite, a cancer cell, a tumor cell, an allergen, or a self-molecule.

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Parent Case Text

CROSS-REFERENCE TO OTHER APPLICATIONS

This application is a national stage of International Patent Application No. PCT/US02/21008, filed Jun. 5, 2002, which claims the benefit of the priority under 35 USC 119(e) of U.S. Provisional Patent Application No. 60/296,531, filed Jun. 7, 2001.

Claims

The invention claimed is:

1. An immunogenic, mutant cholera holotoxin (CT-CRM) comprising an amino acid sequence of subunit A of the ***wild-type*** cholera holotoxin (CT), wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a typtophan or a glycine, wherein the mutant CT-CRM has reduced toxicity compared to ***wild-type*** CT.

2. The CT-CRM according to claim 1, wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a glycine.

3. The CT-CRM according to claim 1, wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a tryptophan.

4. An immunogenic composition comprising an immunogenic, mutant cholera holotoxin (CT-CRM) comprising an amino acid sequence of subunit A of the ***wild-type*** cholera toxin (CT), wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a tryptophan or a glycine, wherein the mutant holotoxin enhances the immune response in a vertebrate host to an antigen.

5. The immunogenic composition according to claim 4, wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a glycine.

6. The composition according to claim 4, further comprising a diluent, excipient or carrier.

7. The composition according to claim 4, further comprising a second adjuvant in addition to the mutant cholera holotoxin.

8. The immunogenic composition according to claim 4, wherein the amino acid arginine in the amino acid position 25 in the A subunit is substituted with a tryptophan.

9. The composition according to claim 4, further comprising an antigen selected from the group consisting of a pathogenic bacterium, a pathogenic virus, a pathogenic fungus and a pathogenic parasite, a cancer cell, a tumor cell, an allergen and a self-molecule, and a protein, polypeptide, peptide or fragment derived from said antigen.

10. The composition according to claim 9, wherein the selected fungal antigen is derived from a fungus selected from the group of pathogenic fungi consisting of Aspergillis, Blastomyces, Candida, Coccidiodes, Cryptococcus and Histoplasma.

11. The composition according to claim 9, wherein the parasite antigen is from a parasite selected from the group of pathogenic parasites consisting of Leishmania major, Ascaris, Trichuris, Giardia, Schistoma, Cryptosporidium, Trichomonas, Toxoplasma gondii and Pneumocystis carinii.

12. The composition according to claim 9, wherein said cancer or tumor cell antigen is selected from the group consisting of prostate specific antigen, carcino-embryonic antigen, MUC-1, Her2, CA-125, MAGE-3, a hormone, and a hormone analogs.

13. The composition according to claim 9, wherein said antigen is a polypeptide, peptide or fragment derived from amyloid precursor protein, or an allergen.

14. The composition according to claim 9, wherein the bacterial antigen is selected from a bacterial species consisting of typable and non-typable Haemophilus influenzae, Haemophilus somnus, Moraxella catarrhalis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus faecalis, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Chlamydia trachomatis, Chlamydia pneumoniae, Chlamydia psittaci, Bordetella pertussis, Alloiococcus otiditis, Salmonella typhi, Salmonella typhimurium, Salmonella choleraesuis, Escherichia coli, Shigella, Vibrio cholerae, Cotynebacterium diptheriae, Mycobacterium tuberculosis, Mycobacterium avium-Mycobacterium intracellular complex, Proteus mirabilis, Proteus vulgaris, Staphylococcus aureus, Staphylococcus epidermis, Clostridium tetani, Leptospira interrogans, Borrelia burdorferi, Pasteurella haemolytica, Pasteurella multocida, Actinobacillus pleauropneumoniae and Mycoplasma galliseptium.

15. The composition according to claim 14, wherein the Haemophilus influenza antigen is selected from the group consisting of the Haemophilus influenzae P4 outer membrane protein, the Haemophilus influenzae P6 outer membrane protein and Haemophilus influenzae adherence and penetration protein (Hap.sub.s).

16. The composition according to claim 15, wherein the Helicobacter Pylon antigen is the Helicobacter pylori urease protein.

17. The composition according to claim 15, wherein the Neisseria meningitidis antigen is selected from the group consisting of the Neisseria meningitidis Group B recombinant class 1 pilin (rpilin) and the Neisseria meningitidis Group B class 1 outer membrane protein (porA).

18. The composition according to claim 9, wherein the viral antigen is selected from the viral species consisting of Respiratory syncytial virus, Parainfluenza virus types 1, 2, 3, Human metapneumovirus, Influenza virus, Herpes simplex virus, Human cytomegalovirus, Human immunodeficiency virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Human papillomavirus, poliovirus, rotavirus, caliciviruses, measles virus, mumps virus, Rubella virus, adenovirus, rabies virus, canine distemper virus, rinderpest virus, avian pneumovirus, Hendra virus, Nipah virus, coronavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis virus and the encephalitis viruses.

19. The composition according to claim 18, wherein the respiratory syncytial virus is the respiratory syncytial virus fusion protein.

20. The composition according to claim 18, wherein the herpes simplex virus (HSV) antigen is the herpes simplex virus (HSV) type 2 glycoprotein D (gD2).

21. The composition according to claim 9, wherein the amyloid precursor protein is the A.beta. peptide, which is a 42 amino acid fragment of amyloid precursor protein, or a fragment of the A.beta. peptide.

Description

BACKGROUND OF THE INVENTION

The body's immune system activates a variety of mechanisms for attacking pathogens (Janeway, Jr, C A. and Travers P., eds., in Immunobiology, "The Immune System in Health and Disease," Second Edition, Current Biology Ltd., London, Great Britain (1996)). However, not all of these mechanisms are necessarily activated after immunization. Protective immunity induced by immunization is dependent on the capacity of an immunogenic composition to, elicit the appropriate immune response to resist or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated and/or humoral immune response.

Many antigens are poorly immunogenic or non-immunogenic when administered by themselves. Strong adaptive immune responses to antigens almost always require that the antigens be administered together with an adjuvant, a substance that enhances the immune response (Audbert, F. M. and Lise, L. D. 1993 Immunology Today, 14: 281-284).

The need for effective immunization procedures is particularly acute with respect to infectious organisms that cause acute infections at, or gain entrance to the body through, the gastrointestinal, pulmonary, nasopharyngeal or genitourinary surfaces. These areas are bathed in mucus, which contains immunoglobulins consisting largely of secretoty immunoglobulin IgA (Hanson, L. A., 1961 Intl. Arch. Allergy. Appl. Immunol., 18, 241-267; Tomasi, T. B., and Zigelbaum, S., 1963 J. Clin. Invest., 42, 1552-1560; and Tomasi, T. B., et al., 1965 J. Exptl. Med., 121, 101-124). This immunoglobulin is derived from large numbers of IgA-producing plasma cells, which infiltrate the larnina propria regions underlying the mucosal membranes (Brandtzaeg, P., and Baklein, K, Scand. 1976 J. Gastroenterol., 11 (Suppl. 36), 1-45; and Brandtzaeg, P., 1984 "Immune Functions of Human Nasal Mucosa and Tonsils in Health and Disease", page 28 et seq. in Immunology of the Lung and Upper Respiratory Tract, Bienenstock, J., ed., McGraw-Hill, New York, N.Y.). The secretory immunoglobulin IgA is specifically transported to the luminal surface through the action of the secretory component (Solari, R, and Kraehenbuhl, J-P, 1985 Immunol. Today, 6, 17-20).

Parenteral immunization regimens are usually ineffective in inducing secretory IgA responses. Secretory immunity is most often achieved through the direct immunization of mucosally associated lymphoid tissues. Following their induction at one mucosal site, the precursors of IgA-producing plasma cells extravasate and disseminate to diverse mucosal tissues where final differentiation to high-rate IgA synthesis occurs (Crabbe, P. A, et al., 1969 J. Exptl. Med., 130, 723-744; Bazin, H., et al., 1970 J. Immunol., 105, 1049-1051; Craig, S. W., and Cebra, J. J., 1971 J. Exptl. Med., 134, 188-200). Extensive studies have demonstrated the feasibility of mucosal immunization to induce this common mucosal immune system (Mestecky, J., et al., 1978 J. Clin. Invest., 61, 731-737). With rare exceptions the large doses of antigen required to achieve effective immunization have made this approach impractical for purified antigens.

Among the strategies investigated to overcome this problem is the use of mucosal adjuvants. A number of adjuvants that enhance the immune response of antigens are known in the prior art (Elson, C. O., and Ealding, W., 1984 J. Immunol., 132, 2736-2741). These adjuvants, when mixed with an antigen, render the antigen particulate, helping retain the antigen in the body for longer periods of time, thereby promoting increased macrophage uptake and enhancing immune response. However, untoward reactions elicited by many adjuvants or their ineffectiveness in inducing mucosal immunity have necessitated the development of better adjuvants for delivery of immunogenic compositions. Unfortunately, adjuvant development to date has been largely an empirical exercise (Janeway, Jr., et al, cited above at pages 12-25 to 12-35). Thus, a rational and a more direct approach is needed to develop effective adjuvants for delivery of antigenic compositions.

It has been reported that the toxin secreted by the Gram-negative bacterium Vibrio cholerae (V. cholerae), the causative agent of the gastrointestinal disease cholera, is extremely potent as an adjuvant. Cholera toxin (CT) has been reported as a 382 amino acid sequence (SEQ ID NO: 1) (Mekalanos, J. J., et al, 1983 Nature, 306, 551-557), which has an 18 amino acid signal (amino acids 1 to 18 of SEQ ID NO: 1). The cholera toxin holotoxin molecule is a hexaheteromeric complex that consists of a single peptide subunit designated CT-A (SEQ ID NO: 2 or amino acids 19 to 258 of SEQ ID NO: 1), which is responsible for the enzymatic activity of the toxin, and five identical peptide subunits, each designated CT-B (each having a 21 amino acid signal (amino acids 259 to 279 of SEQ ID NO:1), followed by the CT-B peptide subunit (amino acids 280 to 382 of SEQ ID NO: 1)), which are involved in the binding of the toxin to the intestinal epithelial cells as well as other cells which contain ganglioside GM.sub.1 on their surface (Gill, D. M., 1976 Biochem., 15, 1242-1248; Cuatrecasas, P., 1973 Biochem., 12, 3558-3566). CT produced by V. cholerae has the CT-A subunit proteolytically cleaved within the single disulfide-linked loop between the cysteines at amino acid positions 187 and 199 of the mature CT-A (SEQ ID NO: 2). This cleavage produces an enzymatically active A1 polypeptide (Kassis, S., et al., 1982 J. Biol. Chem., 257, 12148-12152) and a smaller polypeptide A2, which links fragment A1 to the CT-B pentamer (Mekalanos, J. J., et al., 1979 J. Biol. Chem., 254, 5855-5861). Toxicity results when the enzymatically active fragment CT-A1, upon entry into enterocytes, ADP-ribosylates a regulatory G-protein (Gs.alpha.). This leads to constitutive activation of adenylate cyclase, increased intracellular concentration of cAMP, and secretion of fluid and electrolytes into the lumen of the small intestine (Gill, D. M., and Meren, R, 1978 Proc. Natl. Acad. Sci., USA, 75, 3050-3054), thereby causing toxicity. In vitro, ADP-ribosyl transferase activity of CT is stimulated by the presence of accessory proteins called ARFs, small GTP-binding proteins known to be involved in vesicle trafficking within the eukaryotic cell (Welsh, C. F., et al., "ADP-Ribosylation Factors: A Family of Guanine Nucleotide-Binding Proteins that Activate Cholera Toxin and Regulate Vesicular Transport", pages 257-280 in Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease Vol. 8 (Moss, J., et al., eds., Marcel Dekker, Inc., New York, N.Y. 1995).

Co-administration of CT with an unrelated antigen has been reported to result in the induction of concurrent circulating and mucosal antibody responses to that antigen (Mekalanos, J. J., et al., 1983 Nature, 306, 551-557). To minimize the occurrence of undesirable symptoms such as diarrhea caused by wild-type CT in humans, it would be preferable to use as an adjuvant a form of the CT holotoxin that has substantially reduced toxicity. Mutants of CT have been suggested as a means for achieving a more useful adjuvant. One way to rationally design mutant cholera toxin holotoxins (designated CT-CRMs) with substantially reduced toxicity is to identify and alter amino acid residues in the toxin molecule that are completely conserved in the family of cholera (CT) and related heat-labile enterotoxins (LT-I, LT-IIa and LT-IIb) of E. coli. Another rational way to generate mutant CT-CRMs with substantially reduced toxicity is to alter amino acid residues in the holotoxin molecule that have been identified as being important for NAD-binding based on the structural alignment of the CT backbone with the backbone of related toxins possessing ADP-ribosyl transferase enzyme activity such as diphtheria toxin (DT) and pertussis toxin (PT) (Holmes, R K, "Heat-labile enterotoxins (Escherichia coli)" in Guidebook to Protein Toxins and their Use in Cell Biology, Montecucco, C. and Rappnoli, R., Eds., Oxford Univ. Press, Oxford, England (1997); and Holmes, R. K et al, "Cholera toxins and related enterotoxins of Gram-negative bacteria", pp. 225-256 in Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease, vol. 8, Moss. J., et al, Eds., Marcel Dekker, Inc., New York, N.Y. 1995).

Recently, one such rationally-designed, genetically-detoxified mutant of CT was disclosed wherein a single nonconservative amino acid substitution (glutamic acid to histidine) was introduced by altering the amino acid at position 29 in the mature A subunit (designated CT-CRM.sub.E29H). The resulting mutant cholera holotoxin demonstrated substantially reduced enzymatic toxicity, but with superior adjuvanting and immunogenic properties (International Patent Publication No. WO 00/18434, incorporated in its entirety by reference).

Thus, there is a need to identify and/or rationally design additional mutant forms of the CT holotoxin that have substantially reduced toxicity, yet possess the same or enhanced adjuvanting properties as the wild-type CT holotoxin.

SUMMARY OF THE INVENTION

In one aspect, this invention provides novel mutant, immunogenic forms of cholera holotoxin (designated CT-CRMs) having significantly reduced toxicity compared to wild-type cholera holotoxin (CT), but which retain the ability to function as powerful stimulators of the immune system. Specifically, the invention pertains to five mutant cholera holotoxins (CT-CRMs), desirably generated by site-directed mutagenesis and having substantially reduced toxicity compared to wild-type CT, but with no loss in adjuvanting properties.

In one embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 25 of the A subunit is substituted with another amino acid, which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid arginine at amino acid position 25 of the A subunit is substituted with a tryptophan or a glycine. For determination of the amino acid position, the sequence of CT-A is exemplified in SEQ ID NO: 2. However, other variants and fragments of CT-A may also be employed.

In another embodiment, a novel immunogenic mutant CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein there is an insertion of a single amino acid residue in the amino acid position 49 of the A subunit, which insertion results in a substantial reduction in toxicity. In this aspect and throughout this application, whenever it is stated that "there is an insertion of a single (or multiple) amino acid residue(s) in the A subunit", this means that the wild-type residue(s) in amino acid position(s) [insert amino acid number(s)] is (are) shifted downstream. In a preferred embodiment of the invention, the amino acid residue histidine is inserted in the amino acid position 49 of the A subunit, thereby shifting the amino acid residues originally located at positions 49, 50, etc., to positions 50, 51, etc.

In a third embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein there is an insertion of two amino acid residues in the amino acid positions 35 and 36 in the A subunit,

which insertion results in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid residues glycine and proline are inserted at the amino acid positions 35 and 36 in the A subunit, thereby shifting the original amino acid residues at positions 35 and 36 to positions 37 and 38, etc.

In yet another embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein there is an amino acid substitution in the amino acid residue 30 of the A subunit and an insertion of two amino acid residues in the amino acid positions 31 and 32 in the A subunit, which substitution and insertion results in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid tryptophan is substituted for tyrosine at amino acid position 30 of the A subunit, and the amino acid residues alanine and histidine are inserted in the amino acid positions 31 and 32, respectively, in the A subunit, thereby shifting the original amino acid residues at positions 31 and 32 to positions 33 and 34, etc.

In another aspect, the invention provides a method for producing the novel CT-CRMs described above by employing site-directed mutagenesis of the DNA encoding the A subunit in the wild-type CT using conventional techniques, such that the mutagenized CT now has substantially reduced toxicity without compromising the toxin's ability to stimulate an immune response.

In yet another aspect of the invention, there is provided an immunogenic composition comprising a selected antigen, a mutant CT-CRM as described above as an adjuvant to enhance the immune response in a vertebrate host to the antigen, and a pharmaceutically acceptable diluent, excipient or carrier. Preferably, the CT-CRM is useful for the generation or enhancement of systemic and/or mucosal antigenic immune responses in a vertebrate host to the selected antigen. The selected antigen may be a polypeptide, peptide or fragment derived from a pathogenic virus, bacterium, fungus or parasite. The selected antigen may be a polypeptide, peptide or fragment derived from a cancer cell or tumor cell. The selected antigen may be a polypeptide, peptide or fragment derived from an allergen so as to interfere with the production of IgE so as to moderate allergic responses to the allergen. The selected antigen may be a polypeptide, peptide or fragment derived from a molecular portion thereof which represents those produced by a host (a self molecule) in an undesired manner, amount or location, such as those from amyloid precursor protein so as to prevent or treat disease characterized by amyloid deposition in a vertebrate host.

In one embodiment of this aspect of the invention, there is provided an immunogenic composition selected comprising a selected antigen as described above with a mutant, immunogenic CT-CRM protein of the invention, and a pharmaceutically acceptable diluent, excipient or carrier.

In still another aspect, this invention provides a method for using these CT-CRMs as adjuvants in immunogenic compositions or methods for increasing the ability of an antigenic composition containing a selected antigen as described above to elicit an immune response in vertebrate host by including an effective adjuvanting amount of one or more of the novel detoxified mutant cholera holotoxins (CT-CRMs) described above.

In yet a further aspect of the invention, there are provided DNA sequences encoding the novel immunogenic, mutant CT-CRMs with substantially reduced toxicity as described above. Preferably, the DNA sequence(s) encodes for both the mutant A subunit with reduced toxicity and subunit B. Alternatively, the DNA sequence may encode only the mutant A subunit with reduced toxicity, where the altered or mutant CT-A is fused with an additional binding domain, or is co-expressed with LT-B and allowed to co-assemble.

In a further aspect of the invention, there is provided a plasmid containing isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, detoxified, mutant cholera holotoxin as described herein, and wherein such a DNA sequence is operatively linked to regulatory sequences which direct expression of the CT-CRM in a host cell. Preferably the regulatory sequences comprise an arabinose inducible promoter. In one embodiment of this aspect, the invention relates to a plasmid, designated pLP915, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the amino acid arginine in amino acid position 25 of the A subunit is substituted with an tryptophan. In another embodiment of the invention, the invention relates to a plasmid, designated pLP911, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the amino acid arginine in the amino acid position 25 of the A subunit is substituted with a glycine.

In yet another embodiment of this aspect of the invention, there is provided a plasmid, designated pLP907, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the amino acid residue histidine is inserted in the amino acid position 49 in the A subunit. In still another embodiment of this aspect, the invention relates to a plasmid, designated pLP909, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid residues glycine and proline are inserted in the amino acid positions 35 and 36 in the A subunit. In still a further embodiment, the invention relates to a plasmid, designated pLP910, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid residue tyrosine in amino acid position 30 of the A subunit is substituted with the amino acid residue tryptophan, and the amino acid residues alanine and histidine are inserted in the amino acid positions 31 and 32 in the A subunit.

In a further aspect of the invention, there is provided a suitable host cell line transformed, infected, transduced or transfected with a plasmid as described herein. The immunogenic, detoxified, mutant cholera holotoxins are produced by transforming, infecting, transducing or transfecting a suitable host cell with one of the plasmids described above and culturing the host cell under culture conditions which permit the expression by the host cell of said recombinant immunogenic, mutant cholera holotoxin protein with substantially reduced toxicity.

These and other aspects of the invention will be apparent to one of skill in the art upon reading of the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Mutant forms of cholera holotoxin that exhibit reduced toxicity, but which retain their superior adjuvanting properties, and the utility of these mutant forms of CTs as adjuvants in immunogenic compositions are described herein.

A. Mutant, Detoxified Cholera Toxin Holotoxins

Novel mutant, detoxified immunogenic forms of cholera holotoxin (CT-CRMs) of this invention are characterized by significantly reduced toxicity compared to a wild-type CT. However, such CT-CRMs retain their ability as powerful stimulators of the immune system. The CT-CRMs of this invention are characterized by one or several amino acid substitutions and/or insertions in the mature CT-A subunit of cholera toxin. The various mutant CT-A subunits of this invention also retained their ability to assemble with CT-B subunits to form mutant CT holotoxins that resembled wild-type CT in adjuvanticity, but which exhibited substantially reduced toxicity compared to the wild-type CT. The CT-CRMs of this invention may employ mutant or altered CT-A subunits associated with wild-type CT-B subunits to create a functional holotoxin. Alternatively, the CT-CRMs of this invention may comprise the altered or mutated CT-A subunits

associated with altered or mutated CT-B subunits.

For determination of the amino acid position numbers describing the locations of the amino acid substitutions or insertions in the CT-CRMs of this invention, the sequence of mature CT-A is exemplified as SEQ ID NO: 2, i.e., amino acids 19-258 of SEQ ID NO: 1, a wild-type CT sequence. The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International patent publication No. WO 93/13202. Similarly, a suitable mature CT-B sequence may be illustrated by amino acids 280-382 of SEQ ID NO: 1. However, other variants, biotypes and fragments of CT-A and CT-B of V. cholerae may also be employed as sequences containing the amino acid substitutions and insertions described herein. See, for example, the ELTOR biotype of C. Shi et al, 1993 Sheng Wu Hua Hsueh Tsa Chih, 9(4):395-399; NCBI database locus No. AAC34728, and other sources of variants of V. cholerae toxin.

In one embodiment of this invention, the amino acid substitutions or insertions resulting in some of the CT-CRMs of this invention are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e. conservative amino acid replacements. "Conservative" amino acid substitutions or insertions may be made on the basis of similarity in polarity, charge, solubility hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, tryptophan, and methionine; polar/neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. This invention is exemplified by CT-CRMs, two bearing a single amino acid substitution, one bearing a single amino acid insertion, one bearing a double amino acid insertion, and one bearing a single amino acid substitution and a double amino acid insertion. These CT-CRMs were generated as described in detail in Example 1 with the following mutations in the A subunit as set forth in Table 1.

TABLE-US-00001 TABLE 1 Single and Double CT-CRM Mutants Amino Acid Substitution Native Mutant Abbreviation 25 Arginine Tryptophan CT-CRM.sub.R25W 25 Arginine Glycine CT-CRM.sub.R25G 48 and 49 Threonine.sub.48 Threonine.sub.48, CT-CRM.sub.T48TH Histidine.sub.49 34, 35, 36 Glycine.sub.34 Glycine.sub.34, CT-CRM.sub.G34GGP Glycine.sub.35, Proline.sub.36 30, 31, 32 Tyrosine.sub.30 Tryptophan.sub.30, CT-CRM.sub.Y30WAH Alanine.sub.31, Histidine.sub.32

Thus, in one embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 25 of the A subunit is substituted with another amino acid which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid arginine at amino acid position 25 of the A subunit is substituted with a tryptophan. In another preferred embodiment of the invention, the amino acid arginine at amino acid position 25 of the A subunit is substituted with a glycine. The resulting CT-CRM.sub.R25W and CT-CRM.sub.R25G each demonstrate superior adjuvanting properties.

A novel CT-CRM of this invention comprises a single amino acid insertion at the amino acid position at the amino acid position adjacent to the amino acid residue at the amino acid position 48 in the A subunit, which insertion results in a substantial reduction of toxicity. In a preferred embodiment of the invention the amino acid histidine is inserted adjacent to the amino acid position 48 in the A subunit, resulting in the mutant CT-CRM.sub.T48TH, which demonstrates superior adjuvanting properties.

Another novel CT-CRM of this invention comprises a double amino acid insertion in the amino acid positions 35 and 36 adjacent to the amino acid residue at the amino acid position 34, in the A subunit, which insertion results in a substantial reduction of toxicity. In a preferred embodiment of the invention the amino acids glycine and proline are inserted adjacent to the amino acid position glycine 34 in the A subunit, resulting in the mutant CT-CRM.sub.G34GGP, which demonstrates superior adjuvanting properties.

Yet another novel CT-CRM of this invention comprises a single amino acid substitution at the amino acid position 30 and double amino acid insertion at the amino acid positions 31 and 32 adjacent to the amino acid residue at the amino acid position 30), in the A subunit, which substitution and insertion results in a substantial reduction of toxicity. In a preferred embodiment of the invention, the amino acid residue tyrosine at amino acid position 30 is substituted with the amino acid residue tryptophan and the amino acid residues alanine and histidine are inserted thereafter, resulting in the mutant CT-CRM.sub.Y30WAH, which demonstrates superior adjuvanting properties.

Still other CT-CRMs of this invention may contain at least the single substitutions or single or double mutations described specifically above and at least one additional mutation at a position other than at one or more of the amino acid residues 25, 30, 31, 32, 34, 35, 36, 48 and 49, as set forth above. International patent publication No. WO 93/13202, which is hereby incorporated by reference, describes a series of mutations in the CT-A subunit that serve to reduce the toxicity of the cholera holotoxin. These mutations include making substitutions for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the glutamic acid at position 29, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192. International patent publication No. WO 98/42375, which is hereby incorporated by reference, describes making a substitution for the serine at amino acid 109 in the A subunit, which serves to reduce the toxicity of the cholera holotoxin.

Other useful CT-CRM mutant proteins useful in this invention include a full-length holotoxin with one or more of the specific mutations provided above, and a hexameric, CT-CRM polypeptide or a fragment thereof containing the mutagenized residues described above and which protein, polypeptide or fragment retains the adjuvanticity of wild-type CT from which it is derived, but is characterized by reduced toxicity. Immunologically active fragments of these CT-CRMs with reduced enzymatic activity may also be useful in the methods and compositions of this invention. Fragments ordinarily will contain at least at least about 25 contiguous amino acids of the CT-CRM subunit proteins containing the sites of mutagenesis noted above. More typically a CT-CRM subunit fragment contains at least about 75 contiguous amino acids of the A or B subunits. Another fragment of a CT-CRM subunit contains at least about 100 contiguous amino acids of either subunit. Still another embodiment of a CT-CRM CT-A subunit may contain about 150 amino acids or less than 240 amino acids.

A fragment of the CT-CRMs described herein is useful in the methods and compositions described below if it generates or enhances the immune response to selected antigens in the vertebrate host. Fragments include truncations of the carboxy-terminal region of the CT-CRM subunits. For example, a CT-CRM truncated so that it contains only a CT-A mutant subunit is a desirable fragment. Similarly, CT-A subunits truncated at about residues 240 or 250 are desirable fragments. Still other fragments CT-CRMs of this invention may be selected. Additional fragments of the CT-CRM holotoxin may contain less than five repetitions of the CT-B subunits or truncated CT-B subunits. The foregoing fragments may also contain one or more of the specific mutations described above.

Other suitable CT-CRM proteins may include those in which one or more of the amino acid residues includes a substituted group. Still another suitable CT-CRM holotoxin protein is one in which one or more of the subunits of the hexameric CT-CRM protein is fused with another compound, such as a compound to increase the half-life of the molecule (for example, polyethylene glycol). Another suitable CT-CRM protein is one in which additional amino acids are fused to one or more of the polypeptide subunits, such as a leader or secretory sequence, or a sequence which is employed to enhance the immunogenicity

of the CT-CRM protein. Still other modifications of the CT-CRMs include the above-mentioned deletion of the CT-A signal or leader sequences at the N terminus of CT, i.e., amino acids 1-18 of SEQ ID NO: 1, and/or the deletion of the CT-B signal or leader sequence, i.e., at amino acids 259-279 of SEQ ID NO: 1, and/or the deletion of other regions that do not effect immunogenicity. Similarly, a modification of the CT-CRMs described herein includes include replacing either signal or leader sequences with other signal or leader sequences. See, e.g., U.S. Pat. No. 5,780,601, incorporated by reference herein.

Still another example of suitable CT-CRM proteins are those in which optional amino acids (e.g., -Gly-Ser-) or other amino acid or chemical compound spacers may be included at the termini of the polypeptide subunits for the purpose of lining multiple holotoxin proteins together or to a carrier. For example, useful CT-CRMs may include one or more of the above-described CT-CRMs or subunits thereof coupled to a carrier protein. Alternatively, a useful CT-CRM may be present in a fusion protein containing multiple CT-CRMs, optionally coupled to carrier protein.

For these embodiments, the carrier protein is desirably a protein or other molecule that can enhance the immunogenicity of the selected CT-CRM. Such a carrier may be a larger molecule that also has an adjuvanting effect. Exemplary conventional protein carriers include, without limitation, E. coli DnaK protein, galactokinase (GalK, which catalyzes the first step of galactose metabolism in bacteria), ubiquitin, .alpha.-mating factor, .beta.-galactosidase, and influenza NS-1 protein. Toxoids (i.e., the sequence which encodes the naturally occurring toxin, with sufficient modifications to eliminate its toxic activity) such as diphtheria toxoid and tetanus toxoid, their respective toxins, and any mutant forms of these proteins, such as CRM.sub.197 (a non-toxic form of diphtheria toxin, see U.S. Pat. No. 5,614,382), may also be employed as carriers. Other carriers include exotoxin A of Pseudomonas aeruginosa, heat labile toxins of E. coli and rotaviral particles (including rotavirus and VP6 particles). Alternatively, a fragment or epitope of the carrier protein or other immunogenic protein may be used. For example, a hapten may be coupled to a T cell epitope of a bacterial toxin. See U.S. Pat. No. 5,785,973. Similarly a variety of bacterial heat shock proteins, e.g., mycobacterial hsp-70 may be used. Glutathione-S-transferase (GST) is another useful carrier. One of skill in the art can readily select an appropriate carrier for use in this context. The fusion proteins may be formed by standard techniques for coupling proteinaceous materials. Fusions may be expressed from fused gene constructs prepared by recombinant DNA techniques as described below.

Other suitable CT-CRMs described herein can differ from the specifically exemplified CT-CRMs by modifications that do not revive enzymatic toxicity, and do not diminish adjuventicity, or by combinations of such attributes. Preferably, the amino acid substitutions are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e. conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, tryptophan, and methionine; polar/neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

For example, conservative amino acid changes may be made, which, although they alter the primary sequence of the subunits of the CT-CRM protein, do not normally alter the function of the molecule. In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art (Kyte & Doolittle, 1982, J. Mol. Biol., 157(1):105-32). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still result in a polypeptide with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydropathic character of the amino acid residue determines the secondary and tertiary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydropathic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydropathic indices are within +/-2 is preferred, those within +/-1 are particularly preferred, and those within +/-0.5 are even more particularly preferred.

Substitution or insertion of like amino acids can also be made on the basis of hydrophilicity, particularly where the biologically functional equivalent polypeptide or peptide thereby created is intended for use in immunological embodiments. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a polypeptide, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the polypeptide. As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0.+-.1); glutamate (+3.0.+-.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5.+-.1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within .+-.2 is preferred; those within .+-.1 are particularly preferred; and those within .+-.0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition, modifications, which do not normally alter the primary sequence of the CT-CRM protein, include in vivo or in vitro chemical derivatization of polypeptides, e.g., acetylation, methylation, or carboxylation. Also included as CT-CRMs of this invention are these proteins modified by glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; or by exposing the polypeptide to enzymes which affect glycosylation, such as mammalian glycosylating or deglycosylating enzymes. Also embraced as CT-CRMs are the above-identified mutagenized sequences, which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

Also included as CT-CRMs of this invention are the above sequences that have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties. Among such CT-CRMs are included those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. Among other known modifications which may be present in CT-CRMs of the present invention are, without limitation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

The phenotypic effects of the novel CT-CRMs of Table 1 on the structure and function of CT were assessed. The mutant A subunits with either a single amino acid substitution, a single amino acid insertion, a double amino acid insertion or a single amino acid substitution and double amino acid insertion, generated by site directed mutagenesis of the CT-encoding gene were also able to assemble with CT-B subunits into immunoreactive holotoxin in the presence of subunit B as determined by non-denaturing gel electrophoresis assay (see Table 2, Example 2). Each mutant holotoxin was also tested in a Y-1 adrenal tumor cell assay to determine its residual toxicity compared to wild-type CT holotoxin (see Tables 3 and 4, Example 3). These holotoxins resembled wild-type CT in their adjuvanticities, but the results presented in Table 3 demonstrate that the mutant CT-CRMs had substantially reduced toxicity when compared with wild-type cholera holotoxin. The residual toxicity of the CT-CRMs with single and double amino acid substitutions were substantially reduced in comparison to that of the wild-type CT. These data demonstrate that the mutant CT-CRMs are holotoxins and are substantially less toxic than wild-type CT. Specifically, the mutant CT-CRMs displayed significantly lower levels of toxicity than the wild-type cholera holotoxin in the Y-1 mouse adrenal cell assay.

Each of the mutant CT-CRMs was also compared to wild-type CT in an ADP-ribosyltransferase activity assay (see Example 4). The results, which were generally in agreement with the toxicity data generated in the Y-1 adrenal cell assay, indicated that the ADP-ribosyltransferase activity of the various CT-CRMs was substantially reduced when compared to wild-type CT (Tables 5 and 6).

As used herein, the terms and phrases "the holotoxin has reduced toxicity" or "substantially less toxic" or the like mean that the CT-CRM mutant of this invention, such as the five CT-CRM mutants described herein (CT-CRM.sub.R25W, CT-CRM.sub.R25G, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH), exhibits a substantially lower toxicity per unit of purified toxin protein compared to the wild-type CT. This "reduced toxicity" enables each mutant to be used as an adjuvant in an immunogenic composition without causing significant side effects, particularly those known to be associated with CT, e.g., diarrhea. As described in more detail below, the mutant CT-CRMs of this invention display significantly lower levels of toxicity than the wild-type CT in the Y-1 mouse adrenal cell assay, and a significantly reduced ADP-ribosyltransferase activity when compared to wild-type CT.

The immunogenic mutant CT-CRMs according to the present invention exhibit a balance of reduced toxicity and retained adjuvanticity, such that the resulting mutant CT protein functions as an adjuvant while being tolerated safely by the vertebrate host to which it is introduced. As indicated in the examples below, results in murine model assay systems indicate that the mutant CT-CRMs disclosed herein were able to significantly augment mucosal and systemic immune responses following intranasal administration of disparate antigens. Furthermore, even in the presence of pre-existing anti-CT immune responses, the mutant CT-CRMs were able to serve as efficient mucosal adjuvants. The studies that support these characteristics of the CT-CRMs of this invention are summarized below and more specifically stated in the Examples.

To evaluate the efficacy of the mutant CT-CRMs as mucosal adjuvants for compositions containing bacterial or viral antigens that have been identified as candidates for inclusion in immunogenic compositions, two disparate model antigen systems were examined: (1) the recombinant P4 outer membrane protein (also known as protein "e"(rP4)) of the nontypable Haemophilus influenzae bacterium (NTHi), (see U.S. Pat. No. 5,601,831), and (2) the native UspA2 outer membrane protein of the Moraxella catarrhalis bacterium (International Patent Publication No. WO 98/28333).

Importantly, the data demonstrate that the mutant CT-CRMs are able to augment mucosal and systemic immune responses following intranasal (IN) administration of disparate antigens. Results in murine model systems indicate that all mutant CT-CRMs disclosed herein were able to significantly augment mucosal and systemic immune responses following intranasal administration of these disparate antigens. Furthermore, even in the presence of pre-existing anti-CT immune responses, the mutant CT-CRMs were able to serve as efficient mucosal adjuvants (see Tables 6-18).

The immunogenic mutant CT-CRMs according to the present invention exhibit a balance of reduced toxicity and retained adjuvanticity, such that the protein functions as an adjuvant while being tolerated safely by the vertebrate host immunized with the composition.

B. Nucleic Acid Molecules Encoding CT-CRMs

Another aspect of this invention includes isolated, synthetic or recombinant nucleic acid molecules and sequences encoding the above-described CT-CRMs and/or subunits thereof having the specified site directed mutations, substitutions and/or insertions, or fragments that may further contain one or more of those mutations, substitutions and/or insertions.

An isolated nucleotide molecule comprising a nucleic acid sequence encoding a CT-CRM protein may be preferably under the control of regulatory sequences that direct expression of the CT-CRM in a host cell. As described herein, such nucleic acid molecules may be used to express the CT-CRM protein in vitro or to permit expression of the CT-CRM protein in vivo in a human.

As used herein, the term "isolated nucleotide molecule or sequence" refers to a nucleic acid segment or fragment which is free from contamination with, other biological components that may be associated with the molecule or sequence in its natural environment. For example, one embodiment of an isolated nucleotide molecule or sequence of this invention is a sequence separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, such as the sequences adjacent to the fragment in a genome in which it naturally occurs. Further, the nucleotide sequences and molecules of this invention have been altered to encode a CT-CRM protein of this invention. Thus, the term "isolated nucleic acid molecule or sequence" also applies to nucleic acid sequences or molecules that have been substantially purified from other components that naturally accompany the unmutagenized nucleic acid, e.g., RNA or DNA or proteins, in the cell. An isolated nucleotide molecule or sequence of this invention also encompasses sequences and molecules that have been prepared by other conventional methods, such as recombinant methods, synthetic methods, e.g., mutagenesis, or combinations of such methods. The nucleotide sequences or molecules of this invention should not be construed as being limited solely to the specific nucleotide sequences presented herein, but rather should be construed to include any and all nucleotide sequences which share homology (i.e., have sequence identity) with the nucleotide sequences presented herein.

The terms "substantial homology" or "substantial similarity," when referring to a nucleic acid or fragment thereof, indicate that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 70% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, a program in GCG Version 6.1. The term "homologous" as used herein, refers to the sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a nucleotide or amino acid position in both of the two molecules is occupied by the same monomeric nucleotide or amino acid, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous, then the two sequences are 50% homologous. If 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology. By the term "substantially homologous" as used herein, is meant DNA or RNA which is about 70% homologous, more preferably about 80% homologous, and most preferably about 90% homologous to the desired nucleic acid.

The invention is also directed to an isolated nucleotide molecule comprising a nucleic acid sequence that is at least 70%, 80% or 90% homologous to a nucleic acid sequence encoding a CT-CRM protein or subunit of this invention that has reduced enzymatic toxicity compared to wild-type CT protein and that retains adjuvanticity of the wild-type CT. Furthermore, due to the degeneracy of the genetic code, any three-nucleotide codon that encodes a mutant or substituted amino acid residue of CT-CRM, described herein is within the scope of the invention.

Where, as discussed herein, CT-CRMs, mutant CT-A subunits, or mutant CT-B subunits, and/or DNA sequences encoding them, or other sequences useful in nucleic acid molecules or compositions described herein are defined by their percent homologies or identities to identified sequences, the algorithms used to calculate the percent homologies or percent identities include the following: the Smith-Waterman algorithm (J. F. Collins et al, 1988, Comput. Appl. Biosci., 4:67-72; J. F. Collins et al, Molecular Sequence Comparison and Alignment, (M. J. Bishop et al, eds.) In Practical Approach Series: Nucleic Acid and Protein Sequence Analysis XVIII, IRL Press: Oxford, England, UK (1987) pp. 417), and the BLAST and FASTA programs (E. G. Shpaer et al, 1996, Genomics, 38:179-191). These references are incorporated herein by reference.

By describing two DNAs as being "operably linked" as used herein, is meant that a single-stranded or double-stranded DNA comprises each of the two DNAs and that the two DNAs are arranged within the DNA in such a manner that at least one of the DNA sequences is able to exert a physiological effect by which it is characterized upon the other.

Preferably, for use in producing a CT-CRM protein of this invention or in administering it for in vivo production in a cell, each CT-CRM protein encoding sequence and necessary regulatory sequences are present in a separate viral or non-viral recombinant vector (including non-viral methods of delivery of a nucleic acid molecule into a cell). Alternatively, two or more of these nucleic acid sequences encoding duplicate copies of a CT-CRM protein or encoding multiple different CT-CRMs of this invention may be contained in a polycistronic transcript, i.e., a single molecule designed to express multiple gene products.

The invention further relates to vectors, particularly plasmids, containing isolated and purified DNA sequences comprising DNA sequences that encode an immunogenic mutant cholera holotoxin. Desirable embodiments include plasmids containing DNA sequences which encode, for example, an immunogenic mutant cholera holotoxin having single amino acid substitutions at amino acid residue 25 of CT-A, a single amino acid insertion between amino acid residues 48 and 49 of CT-A, double amino acid insertions between amino acid residues 34 and 35 of CT-A, or a single amino acid substitution at amino acid residues 30 and a double amino acid insertion between amino acids 30 and 31 of CT-A By the term "vector" as used herein, is meant a DNA molecule derived from viral or non-viral, e.g., bacterial, species that has been designed to encode an exogenous or heterologous nucleic acid sequence. Thus, the term includes conventional bacterial plasmids. Such plasmids or vectors can include plasmid sequences from viruses or phages. Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses. Vectors may also be derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids, and phagemids. The term also includes non-replicating viruses that transfer a gene from one cell to another. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds and the like.

The nucleic acid molecules of the invention include non-viral vectors or methods for delivery of the sequences encoding the CT-CRM protein to a host cell according to this invention. A variety of non-viral vectors are known in the art and may include, without limitation, plasmids, bacterial vectors, bacteriophage vectors, "naked" DNA and DNA condensed with cationic lipids or polymers.

Examples of bacterial vectors include, but are not limited to, sequences derived from bacille Calmette Guerin (BCG), Salmonella, Shigella, E. coli, and Listeria, among others. Suitable plasmid vectors include, for example, pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pK37, pKC101, pAC105, pVA51, pKH47, pUB110, pMB9, pBR325, Col E1, pSC101, pBR313, pML21, RSF2124, pCR1, RP4, pBAD18, and pBR328.

Examples of suitable inducible Escherichia coli expression vectors include pTrc (Amann et al., 1988 Gene, 69:301-315), the arabinose expression vectors (e.g., pBAD18, Guzman et al, 1995 J. Bacteriol., 177:4121-4130), and pETIId (Studier et al., 1990 Methods in Enzymology, 185:60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pETIId vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase T7 gn 1. This viral polymerase is supplied by host strains BL21 (DE3) or HMS I 74(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV5 promoter. The pBAD system relies on the inducible arabinose promoter that is regulated by the araC gene. The promoter is induced in the presence of arabinose.

As one example, a plasmid, designated pLP9911, contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity having a single amino acid substitution (arginine to tryptophan) at amino acid position 25 in the A subunit (CT-CRM.sub.R25W). As another example, a plasmid, designated pLP9915, contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity having a single amino acid substitution (arginine to glycine) at amino acid position 25 in the A subunit (CT-CRM.sub.R25G). A third plasmid, designated pLP9907, contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein a single amino acid histidine is inserted at the amino acid position 49 adjacent to the amino acid residue threonine at the amino acid position 48 in the A subunit (CT-CRM.sub.T48TH). Another exemplary plasmid is designated pLP9909. This plasmid contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein a double amino acid insertion of amino acid residues glycine and proline is inserted in the amino acid positions 35 and 36 adjacent to the amino acid residue glycine at the amino acid position 34 in the A subunit (CT-CRM.sub.G34GGP). Another plasmid exemplified in this invention is designated pLP9910. It contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein a single amino acid substitution at the amino acid position 30 (substitution of the amino acid residue tyrosine at amino acid position 30 with amino acid residue tryptophan) and a double amino acid insertion of amino acid residues (alanine and histidine) in the amino acid positions 31 and 32 adjacent to the amino acid residue at the amino acid position 30 are made in the A subunit (CT-CRM.sub.Y30WAH).

Another type of useful vector is a single or double-stranded bacteriophage vector. For example, a suitable cloning vector includes, but is not limited to the vectors such as bacteriophage .lamda. vector system, .lamda.gt11, .mu.gt .mu.WES.tB, Charon 4, .lamda.gt-WES-.lamda.B, Charon 28, Charon 4A, .lamda.gt-1-.lamda.BC, .lamda.gt-1-.lamda.B, M13 mp7, M13 mp8, or M13 mp9, among others.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in a yeast such as S. cerevisiae include pYepSec I (Baldari, et al., 1987 Protein Eng., 1(5):433-437), pMFa (Kurjan and Herskowitz, 1982 Cell, 30(3):933-943), pJRY88 (Schultz et al., 1987 Gene, 61(2): 123-133), and pYES2 (Invitrogen Corporation, San Diego, Calif.).

Alternatively, baculovirus expression vectors are used. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 or Sf 21 cells) include the pAc series (Smith et al., 1983 Biotechnol., 24:434-443) and the pVL series (Luckow and Summers, 1989 Virol., 170(1):31-39).

In yet another embodiment, a mammalian expression vector is used for expression in mammalian cells. Examples of mammalian expression vectors include pCDM8 (Seed, 1987 Nature, 329:840-842) and pMT2PC (Kaufman et al., 1987 EMBO J., 6(1):187-93). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements.

One type of recombinant vector is a recombinant single or double-stranded RNA or DNA viral vector. A variety of viral vector systems are known in the art. Examples of such vectors include, without limitation, recombinant adenoviral vectors, herpes simplex virus (HSV)-based vectors, adeno-associated viral (AAV) vectors, hybrid adenoviral/AAV vectors, recombinant retroviruses or lentiviruses, recombinant poxvirus vectors, recombinant vaccinia virus vectors, SV-40 vectors, insect viruses such as baculoviruses, and the like that are constructed to carry or express a selected nucleic acid composition of interest.

Retrovirus vectors that can be employed include those described in EP 0 415 731; International Patent Publication Nos. WO 90/07936; WO 94/03622; WO 93/25698; and WO 93/25234; U.S. Pat. No. 5,219,740; International Patent Publication Nos. WO 93/11230 and WO 93/10218; Vile and Hart, 1993 Cancer Res. 53:3860-3864; Vile and Hart, 1993 Cancer Res. 53:962-967; Ram et al., 1993 Cancer Res. 53:83-88; Takamiya et al., 1992 J. Neurosci. Res. 33:493-503; Baba et al., 1993 J. Neurosurg. 79:729-735; U.S. Pat. No. 4,777,127; GB Patent No. 2,200,651; and EP 0 345 242. Examples of suitable recombinant retroviruses include those described in International Patent Publication No. WO 91/02805.

Alphavirus-based vectors may also be used as the nucleic acid molecule encoding the CT-CRM protein. Such vectors can be constructed from a wide variety of alphaviruses, including, for example, Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR 1249; ATCC VR-532). Representative examples of such vector systems include those described in U.S. Pat. Nos. 5,091,309; 5,217,879; and 5,185,440; and International Patent Publication Nos. WO 92/10578; WO 94/21792; WO 95/27069; WO 95/27044; and WO 95/07994.

Examples of adenoviral vectors include those described by Berkner, 1988 Biotechniques 6:616-627; Rosenfeld et al., 1991 Science 252:431-434; International Patent Publication No. WO 93/19191; Kolls et al., 1994 PNAS 91:215-219; Kass-Eisler et al., 1993 PNAS 90:11498-11502; Guzman et al., 1993 Circulation 88:2838-2848; Guzman et al., 1993 Cir. Res. 73:1202-1207; Zabner et al., 1993 Cell 75:207-216; Li et al., 1993 Hum. Gene Ther. 4:403-409; Cailaud et al., 1993 Eur. J. Neurosci. 5:1287-1291; Vincent et al., 1993 Nat. Genet. 5:130-134; Jaffe et al., 1992 Nat. Genet. 1:372-378; and Levrero et al., 1991 Gene 101: 195-202. Exemplary adenoviral vectors include those described in International Patent Publication Nos. WO 94/12649; WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655. Other adenoviral vectors include those derived from chimpanzee adenoviruses, such as those described in U.S. Pat. No. 6,083,716.

Another viral vector is based on a parvovirus such as an adeno-associated virus (AAV). Representative examples include the AAV vectors described in International Patent Publication No. WO 93/09239, Samulski et al., 1989 J. Virol. 63:3822-3828; Mendelson et al., 1988 Virol. 166:154-165; and Flotte et al., 1993 PNAS 90:10613-10617. Other particularly desirable AAV vectors include those based upon AAV1; see, International Patent Publication No. WO 00/28061, published May 18, 2000. Other desirable AAV vectors include those which: are pseudotyped, i.e., contain a minigene composed of AAV 5' ITRs, a transgene, and AAV 3' ITRs packaged in a capsid of an AAV serotype heterologous to the AAV ITRs. Methods of producing such pseudotyped AAV vectors are described in detail in International Patent Publication No. WO01/83692.

In an embodiment in which the nucleic acid molecule of the invention is "naked DNA", it may be combined with polymers including traditional polymers and non-traditional polymers such as cyclodextrin-containing polymers and protective, interactive noncondensing polymers, among others. The "naked" DNA and DNA condensed with cationic lipids or polymers are typically delivered to the cells using chemical methods. A number of chemical methods are known in the art for cell delivery and include using lipids, polymers, or proteins to complex with DNA, optionally condensing the same into particles, and delivering to the cells. Another non-viral chemical method includes using cations to condense DNA, which is then placed in a liposome and used according to the present invention. See, C. Henry, 2001 Chemical and Engineering News, 79(48):35-41.

The nucleic acid molecule encoding the CT-CRM of this invention is introduced directly into the cells either as "naked" DNA (U.S. Pat. No. 5,580,859) or formulated in compositions with agents that facilitate immunization, such as bupivicaine and other local anesthetics (U.S. Pat. No. 6,127,170).

All components of the viral and non-viral vectors above may be readily selected from among known materials in the art and available from the pharmaceutical industry. Selection of the vector components and regulatory sequences are not considered a limitation on this invention. Each nucleic acid sequence encoding a CT-CRM protein according to this invention is preferably under the control of regulatory sequences that direct the replication and generation of the product of each nucleic acid sequence in a mammalian or vertebrate cell. By the term "promoter/regulatory sequence" is meant a DNA sequence required for expression of a nucleic acid operably linked thereto. Preferably the promoter/regulatory sequence is positioned at the 5' end of the coding sequence such that it drives expression of the CT-CRM protein in a cell. In some instances, the promoter/regulatory sequence may function in a tissue specific manner. For example, the promoter/regulatory sequence is only capable of driving expression in a cell of a particular tissue type. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements that are required for expression in a tissue-specific manner.

Suitable promoters may be readily selected from among constitutive promoters, inducible promoters, tissue-specific promoters and others. Examples of constitutive promoters that are non-specific in activity and employed in the nucleic acid molecules encoding the CT-CRM protein of this invention include, without limitation, the retroviral Rous sarcoma virus (RSV) promoter, the retroviral LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (see, e.g., Boshart et al, Cell, 41:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the .beta.-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1.alpha. promoter (Invitrogen).

Inducible promoters that are regulated by exogenously supplied compounds, include, without limitation, the arabinose promoter, the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, 1996 Proc. Natl. Acad. Sci. USA, 93:3346-3351), the tetracycline-repressible system (Gossen et al, 1992 Proc. Natl. Acad. Sci. USA, 89:5547-5551), the tetracycline-inducible system (Gossen et al, 1995 Science, 268:1766-1769, see also Harvey et al, 1998 Curr. Opin. Chem Biol, 2:512-518), the RU486-inducible system (Wang et al, 1997 Nat. Biotech., 15:239-243 and Wang et al, 1997 Gene Ther., 4:432-441) and the rapamycin-inducible system (Magari et al, 1997 J. Clin. Invest., 100: 2865-2872). A particularly preferred promoter for use in expression systems for CT-CRMs is an arabinose inducible promoter.

Other types of inducible promoters that may be useful in this context are those regulated by a specific physiological state, e.g., temperature or acute phase or in replicating cells only. Useful tissue-specific promoters include the promoters from genes encoding skeletal .beta.-actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally-occurring promoters (see Li et al., 1999 Nat. Biotech., 17:241-245). Examples of promoters that are tissue-specific are known for liver (albumin, Miyatake et al. 1997 J. Virol., 71:5124-32; hepatitis B virus core promoter, Sandig et al., 1996 Gene Ther., 3: 1002-9; alpha-fetoprotein (AFP), Arbuthnot et al., 1996 Hum. Gene Ther., 7:1503-14), bone (osteocalcin, Stein et

al., 1997 Mol. Biol. Rep., 24:185-96; bone sialoprotein, Chen et al., 1996 J. Bone Miner. Res., 11:654-64), lymphocytes (CD2, Hansal et al., 1988 J. Immunol., 161:1063-8; immunoglobulin heavy chain; T cell receptor .alpha. chain), neuronal (neuron-specific enolase (NSE) promoter, Andersen et al. 1993 Cell. Mol. Neurobiol., 13:503-15; neurofilament light-chain gene, Piccioli et al., 1991 Proc. Natl. Acad. Sci. USA, 88:5611-5; the neuron-specific ngf gene, Piccioli et al., 1995 Neuron, 15:373-84); among others. See, e.g., International Patent Publication No. WO00/55335 for additional lists of known promoters useful, in this context.

Additional regulatory sequences for inclusion in a nucleic acid sequence, molecule or vector of this invention include, without limitation, an enhancer sequence, a polyadenylation sequence, a splice donor sequence and a splice acceptor sequence, a site for transcription initiation and termination positioned at the beginning and end, respectively, of the polypeptide to be translated, a ribosome binding site for translation in the transcribed region, an epitope tag, a nuclear localization sequence, an IRES element, a Goldberg-Hogness "TATA" element, a restriction enzyme cleavage site, a selectable marker and the like. Enhancer sequences include, e.g., the 72 bp tandem repeat of SV40 DNA or the retroviral long terminal repeats or LTRs, etc. and are employed to increase transcriptional efficiency. Selection of promoters and other common vector elements are conventional and many such sequences are available with which to design the nucleotide molecules and vectors useful in this invention. See, e.g., Sambrook et al, Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, New York, (1989) and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York (1989). One of skill in the art may readily select from among such known regulatory sequences to prepare molecules of this invention. The selection of such regulatory sequences is not a limitation of this invention.

C. Methods for Making the CT-CRM Proteins and Nucleotide Molecules of this Invention

In view of the demonstrated utility of mutant CT-CRMs as adjuvants for antigenic compositions, production of suitable quantities of mutant CT-CRMs is desirable. The preparation or synthesis of the nucleotide sequences and CT-CRMs, as well as compositions containing the nucleotide molecules or CT-CRM protein of this invention disclosed herein is well within the ability of the person having ordinary skill in the art using available material. The synthesis methods are not a limitation of this invention. The examples below detail presently preferred embodiments of synthesis of sequences encoding the CT-CRMs of this invention.

The CT-CRMs and nucleotide molecules and sequences of this invention may be produced by chemical synthesis methods, recombinant genetic engineering methods, site directed mutagenesis, among others, and combinations of such methods. For example, the nucleotide sequences/CT-CRMs of the invention may be prepared conventionally by resort to known chemical synthesis techniques, e.g., solid-phase chemical synthesis, such as described by Merrifield, 1963 J. Amer. Chem. Soc., 85:2149-2154; J. Stuart and J. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, Ill. (1984); Matteucci et al., 1981 J. Am. Chem. Soc., 103:3185; Alvarado-Urbina et al., 1980 Science, 214:270; and Sinha, N. D. et al., 1984 Nucl. Acids Res., 13:4539, among others. See, also, e.g., PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., "Posttranslational Protein Modifications: Perspectives and Prospects", pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al, 1990 Meth. Enzymol., 182:626-646, and Rattan et al, 1992 Ann. N.Y. Acad. Sci., 663:48-62.

Alternatively, compositions of this invention may be constructed recombinantly using conventional molecular biology techniques, site-directed mutagenesis, genetic engineering or polymerase chain reaction, such as, by cloning and expressing a nucleotide molecule encoding a CT-CRM protein with optional other immunogens and optional carrier proteins within a host microorganism, etc. utilizing the information provided herein (See, e.g., Sambrook et al., cited above; Ausubel et al. cited above). Coding sequences for the CT-CRMs and optional immunogens can be prepared synthetically (W. P. C. Stemmer et al, 1995 Gene, 164:49).

In general, recombinant DNA techniques involve obtaining by synthesis or isolation a DNA sequence that encodes the CT-CRM protein as described above, and introducing it into an appropriate vector/host cell expression system where it is expressed preferably under the control of an arabinose inducible promoter. Any of the methods described for the insertion of DNA into an expression vector may be used to ligate a promoter and other regulatory control elements into specific sites within the selected recombinant vector. Suitable host cells are then transformed, infected, transduced or transfected with such vectors or plasmids by conventional techniques.

A variety of host cell-vector (plasmid) systems may be used to express the immunogenic mutant cholera holotoxin. The vector system, which preferably includes the arabinose inducible promoter, is compatible with the host cell used. The DNA encoding the mutant CT-CRMs are inserted into an expression system, and the promoter (preferably the arabinose inducible promoter), and other control elements are ligated into specific sites within the vector so that when the vector is inserted into a host cell (by transformation, transduction or transfection, depending on the host cell-vector system used) the DNA encoding the CT-CRM is expressed by the host cell.

The vector may be selected from one of the viral vectors or non-viral vectors described above but must be compatible with the host cell used. The recombinant DNA vector may be introduced into appropriate host cells (bacteria, virus, yeast, mammalian cells or the like) by transformation, transduction or transfection (depending upon the vector/host cell system). Host-vector systems include but are not limited to bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); and insect cell systems infected with virus (e.g., baculovirus).

Systems for cloning and expressing the CT-CRMs and other compositions of this invention using the synthetic nucleic acid molecules include the use of various microorganisms and cells that are well known in recombinant technology. The host cell may be selected from any biological organism, including prokaryotic (e.g., bacterial) cells and eukaryotic cells, including, mammalian, insect cells, yeast cells. Preferably, the cells employed in the various methods and compositions of this invention are bacterial cells. Suitable bacterial cells include, for example, various strains of E. coli, Bacillus, and Streptomyces. Yeast cells such as Saccharomyces and Pichia, and insect cells such as Sf9 and Sf21 cells are also useful host cells for production purposes. Mammalian cells including but not limited to Chinese hamster ovary cells (CHO), chick embryo fibroblasts, baby hamster kidney cells, NIH3T3, PER C6, NSO, VERO or COS cells are also suitable host cells, as well as other conventional and non-conventional organisms and plants.

The selection of other suitable host cells and methods for transformation, culture, amplification, screening and product production and purification can be performed by one of skill in the art by reference to known techniques. See, e.g., Gething and Sambrook, 1981 Nature, 293:620-625, among others.

Typically, the host cell is maintained under culture conditions for a period of time sufficient for expression. Culture conditions are well known in the art and include ionic composition and concentration, temperature, pH and the like. Typically, transfected cells are maintained under culture conditions in a culture medium Suitable media for various cell types are well known in the art. In a preferred embodiment, temperature is from about 20.degree. C. to about 50.degree. C., more preferably from about 30.degree. C. to about 40.degree. C. and, even more preferably about 37.degree. C.

The pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. Osmolality is preferably from about 200 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably from about 290 mosm/L to about 310 mosm/L. Other biological conditions needed for transfection and expression of an encoded protein are well known in the art.

Recombinant CT-CRM protein is recovered or collected either from the host cells or membranes thereof or from the medium in which those cells are cultured. Recovery comprises isolating and purifying the recombinant CT-CRM protein. Isolation and purification techniques for polypeptides are well known in the art and include such procedures as precipitation, filtration, chromatography, electrophoresis and the like.

When produced by conventional recombinant means, CT-CRMs of this invention may be isolated and purified from the cell or medium thereof by conventional methods, including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard techniques for the purification or proteins. Several techniques exist for purification of heterologous protein from prokaryotic cells. See, U.S. Pat. Nos. 4,518,526; 4,599,197; and 4,734,362. The purified preparation however produced should be substantially free of host toxins, which might be harmful to humans. In particular, when expressed in gram negative bacterial host cells such as E coli, the purified peptide or protein should be substantially free of endotoxin contamination. See, e.g., Sambrook et al., cited above.

The CT-CRMs used in methods and compositions of the invention are not limited to products of any of the specific exemplary processes listed herein. In fact, the protein may be prepared by the methods in the texts cited immediately above or by methods of the texts cited elsewhere in this specification It is within the skill of the art to isolate and produce recombinantly or synthetically protein compositions for such use.

The five exemplary CT-CRMs of Table 1, two bearing a single amino acid substitution, one bearing a single amino acid insertion, and two bearing double amino acid substitutions were generated as described in detail in Example 1 using some of the methods described above. Specifically, a set of mutant CT clones (CT-CRMs) were generated in E. coli by standard site-directed mutagenesis protocols on plasmids encoding the known CT holotoxin molecules. It has previously been shown that the resulting yield of purified CT-CRM.sub.E29H holotoxin was approximately 50 .mu.g per liter of culture medium (see International patent publication No. WO 00/18434). Initial attempts to increase CT-CRM.sub.E29H yield via modifications to the original plasmid, showed little or no effect. A moderate increase in yield was achieved through co-expression of the plasmid pIIB29H, and derivatives, with Vibrio cholerae DsbA and E. coli RpoH. Co-expression and purification modifications increased the yield of CT-CRM.sub.E29H to approximately 2 mg/liter.

In order to increase the expression of CT-CRMs of the present invention, the lactose inducible promoter in the plasmids was replaced with an arabinose inducible promoter (Invitrogen Corporation, Carlsbad, Calif.), which was operatively linked to the DNA sequence encoding the CT-CRMs. During cloning it was determined that plasmid pIIB29H contained a ctxA gene encoding CT subunit A from Vibrio cholerae strain 569B, linked to a ctxB gene encoding CT subunit B from Vibrio cholerae strain 2125. Cross alignment of these genes indicated seven base substitutions between the two ctxB genes and a single base change between the ctxA genes. Several of these base substitutions led to amino acid changes in the mature subunits. Of special note is the substitution between the ctxA genes which leads to an amino acid change within the A-2 portion, or the holotoxin assembly domain of the A subunit. It was not known whether the heterogeneity between these genes had a negative impact on toxin expression or holotoxin assembly. However, it was thought preferable from an evolutionary standpoint that both toxin subunit genes originate from the same source. As such, both the ctxA and ctxB genes used in the construction of the arabinose inducible system originated from Vibrio cholerae strain 569B. The construction of plasmids pLP911, pLP915, pLP907, pLP909 and pLP910 is described in Example 1. The immunogenic mutant cholera holotoxin is produced by transforming, infecting, transducing or transfecting a host cell with a plasmid described above, and culturing the host cell under conditions that permit the expression of said recombinant immunogenic detoxified protein by the host cell. The yield of CT-CRMs from pLP911, pLP915, pLP907, pLP909 and pLP910 is approximately 7.6, 5.6, 7.9, 27.4, and 1.9 mg of purified material per liter of culture, respectively.

The resulting CT-CRM protein or nucleic acid molecule may be formulated into an immunogenic composition with any number of selected antigens and screened for adjuvant efficacy by in vivo assays, such as those described in the examples below.

D. Immunogenic Compositions

An effective immunogenic composition according to the invention is one comprising a mutant cholera holotoxin of this invention. Preferably the mutant cholera holotoxin CT-CRM has reduced toxicity compared to a wild-type cholera holotoxin. This "reduced toxicity" enables each mutant to be used as an adjuvant in an immunogenic composition without causing significant side effects, particularly those known to be associated with wild-type CT, e.g., diarrhea. More preferably, the CT-CRM in the immunogenic composition of this invention has a single amino acid substitution (arginine to tryptophan or arginine to glycine) at amino acid position 25 in the A subunit (CT-CRM.sub.R25W, CT-CRM.sub.R25G). In another preferred embodiment, the CT-CRM has a single amino acid insertion of histidine in the amino acid position 49 adjacent to the amino acid residue threonine at the amino acid position 48 in the A subunit (CT-CRM.sub.T48TH). A third preferred embodiment is a CT-CRM with a double amino acid insertion of amino acid residues glycine and proline in the amino acid positions 35 and 36 adjacent to the amino acid residue glycine at the amino acid position 34 in the A subunit (CT-CRM.sub.G34GGP). A fourth exemplary CT-CRM has a single amino acid substitution at the amino acid position 30 (tyrosine for tryptophan) and a double amino acid insertion of amino acid residues alanine and histidine in the amino acid positions 31 and 32 adjacent to the amino acid residue at the amino acid position 30 in the A subunit (CT-CRM.sub.Y30WAH). In one embodiment, the CT-CRM may have one or more additional modifications as described above. In another embodiment, the composition comprises a selected antigen and a suitable effective adjuvanting amount of the CT-CRM, wherein said holotoxin significantly enhances the immune response in a vertebrate host to said antigen. The compositions of the present invention modulate the immune response by improving the vertebrate host's antibody response and cell-mediated immune responses to the administration of a composition comprising a selected antigen as described above.

As used herein, the term "effective adjuvanting amount" means a dose of one of the CT-CRM mutants of this invention that is effective in eliciting an increased immune response in a vertebrate host. In a more specific definition, the term "effective adjuvanting amount" means a dose of one of the five CT-CRM mutants described herein (CT-CRM.sub.R25W, CT-CRM.sub.R25G, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH), effective in eliciting an increased immune response in a vertebrate host. Specifically, the CT-CRMs disclosed herein augment mucosal and systemic immune responses following intranasal administration of disparate antigens. Furthermore, even in the presence of pre-existing anti-CT immune responses, the mutant CT-CRMs were able to serve as efficient mucosal adjuvants. The immunogenic mutant CT-CRMs according to the present invention exhibit a balance of reduced toxicity and retained adjuvanticity, such that the resulting mutant CT protein functions as an adjuvant while being tolerated safely by the vertebrate host to which it is introduced. The particular "effective adjuvanting dosage or amount" will depend upon the age, weight and medical condition of the host, as well as on the method of administration Suitable doses are readily determined by persons skilled in the art.

The immunogenic compositions containing as an adjuvant the mutant cholera holotoxins of this invention also contain at least one antigen selected from among a wide variety of antigens. The antigen(s) may comprise a whole cell or virus, or one or more saccharides, proteins, protein subunits, polypeptide, peptide or fragments, poly- or oligonucleotides, or other macromolecular components. If desired, the antigenic compositions may contain more than one antigen from the same or different pathogenic microorganisms.

Thus, in one embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic bacterium. Desirable bacterial immunogenic compositions including the CT-CRM mutant(s) as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, Haemophilus influenzae (both typable and nontypable), Haemophilus somnus, Moraxella catarralis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus faecalis, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Chlamydia trachomatis, Chlamydia pneumoniae, Chlamydia psittaci, Bordetella pertussis, Alloiococcus otiditis, Salmonella typhi, Salmonella typhimurium, Salmonella choleraesuis, Escherichia coli, Shigella, Vibrio cholerae, Corynebacterium diphtheriae, Mycobacterium tuberculosis, Mycobacterium avium-Mycobacterium intracellulare complex, Proteus mirabilis, Proteus vulgaris, Staphylococcus aureus, Staphylococcus epidermidis, Clostridium tetani, Leptospira interrogans, Borrelia burgdorferi, Pasteurella haemolytica, Pasteurella multocida, Actinobacillus pleuropneumoniae and Mycoplasma gallisepticum.

In another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic virus. Desirable viral immunogenic compositions including the CT-CRM mutant(s) as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, Respiratory syncytial virus, Parainfluenza virus types 1-3, Human metapneumovirus, Influenza virus, Herpes simplex virus, Human cytomegalovirus, Human immunodeficiency virus, Simian immunodeficiency virus, Hepatitis A v Hepatitis B virus, Hepatitis C virus, Human papillomavirus, Poliovirus, rotavirus, caliciviruses, Measles virus, Mumps virus, Rubella virus, adenovirus, rabies virus, canine distemper virus, rinderpest virus, avian pneumovirus (formerly turkey rhinotracheitis virus), Hendra virus, Nipah virus, coronavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis virus and various Encephalitis viruses.

In another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic fungus. Desirable immunogenic compositions against fungal pathogens including the CT-CRM mutant(s) as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, Aspergillis, Blastomyces, Candida, Coccidiodes, Cryptococcus and Histoplasma.

In still another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic parasite. Desirable immunogenic compositions against parasites including the CT-CRM mutant(s) as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, Leishmania major, Ascaris, Trichuris, Giardia, Schistosoma, Cryptosporidium, Trichomonas, Toxoplasma gondii and Pneumocystis carinii.

Desirable immunogenic compositions directed against non-infectious diseases including the CT-CRM mutant(s) as an adjuvant are also within the scope of this invention. Such immunogenic compositions include those directed to vertebrate antigens, particularly compositions directed against antigens for the prevention and/or treatment of disease(s), without limitation, such as allergy, autoimmune disease, Alzheimer disease and cancer.

For example, the immunogenic composition of this invention may contain a polypeptide, peptide or fragment derived from a cancer cell or tumor cell. Desirable immunogenic compositions for eliciting a therapeutic or prophylactic anti-cancer effect in a vertebrate host, which contain the CT-CRM mutants of this invention, include those utilizing a cancer antigen or tumor-associated antigen including, without limitation, prostate specific antigen, carcino-embryonic antigen, MUC-1, Her2, CA-125, MAGE-3, hormones, hormone analogs and so forth.

Other immunogenic compositions of this invention are desirable for moderating responses to allergens in a vertebrate host. Such compositions contain the CT-CRM mutant(s) of this invention and a polypeptide, peptide or fragment derived from an allergen or fragment thereof. Examples of such allergens are described in the U.S. Pat. No. 5,830,877 and International patent publication No. WO 99/51259, which are hereby incorporated by reference, and include pollen, insect venoms, animal dander, fungal spores and drugs (such as penicillin). The immunogenic compositions interfere with the production of IgE antibodies, a known cause of allergic reactions, so as to moderate allergic responses to the allergen.

In still another embodiment, the immunogenic compositions of this invention contain as the selected antigen a polypeptide, peptide or fragment derived from a molecular portion of an antigen, which represents those produced by a host (a self molecule) in an undesired manner, amount or location, such as those from amyloid precursor protein so as to prevent or treat disease characterized by amyloid deposition in a vertebrate host. Desirable compositions for moderating responses to self molecules in a vertebrate host, which contain CT-CRM mutants of this invention, include those containing a self molecule or fragment thereof. Examples of such self molecules include .beta.-chain insulin involved in diabetes, the G17 molecule involved in gastroesophageal reflux disease, and antigens which downregulate autoimmune responses in diseases such as multiple sclerosis, lupus and rheumatoid arthritis.

Still other immunogenic compositions of this invention are desirable for preventing or treating disease characterized by amyloid deposition in a vertebrate host. Such compositions contain the CT-CRM mutant(s) of this invention as well as portions of amyloid precursor protein (APP). This disease is referred to variously as Alzheimer's disease, amyloidosis or amyloidogenic disease. The .beta.-amyloid precursor protein (also referred to as A.beta. peptide) is a 42 amino acid fragment of APP, which is generated by processing of APP by the .beta. and .gamma. secretase enzymes, and has the following sequence: Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala (SEQ ID NO: 3). In some patients, the amyloid deposit takes the form of an aggregated A.beta. peptide. Surprisingly, it has now been found that administration of isolated A.beta. peptide induces an immune response against the A.beta. peptide component of an amyloid deposit in a vertebrate host (International patent publication No. WO 99/27944). Thus, embodiments of this invention include the CT-CRM mutants of this invention plus A.beta. peptide, as well as fragments of A.beta. peptide and antibodies to A.beta. peptides or fragments thereof. One such fragment of A.beta. peptide is the 28 amino acid peptide having the following sequence (U.S. Pat. No. 4,666,829): Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys (SEQ ID NO: 4).

Such immunogenic compositions further comprise an immunologically acceptable diluent or a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. The antigenic compositions may also be mixed with such diluents or carriers in a conventional manner. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with administration to humans or other vertebrate hosts. The appropriate carrier will be evident to those skilled in the art and will depend in large part upon the route of administration.

The immunogenic compositions may also include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. Other parenterally-administrable formulations, which are useful, include those, which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer system. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble

salt.

Still additional components that may be present in the protein immunogenic compositions of this invention are adjuvants in addition to the CT-CRMs, preservatives, chemical stabilizers, or other antigenic proteins. Typically, stabilizers, adjuvants, and preservatives are optimized to determine the best formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients that may be used include, for example, casamino acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk.

The antigenic compositions of this invention may comprise further adjuvants in addition to the mutant CT-CRMs. Conventional non-CT-CRM adjuvants used to enhance an immune response include, without limitation, MPL.TM. (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, Mont.), which is described in U.S. Pat. No. 4,912,094, which is hereby incorporated by reference. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, Mont.), and which are described in U.S. Pat. No. 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino]ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyoxytetradecanoyl]-2-[(R)-3-t- etradecanoyoxytetradecanoyl-amino]-b-D-glucopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form or as a stable emulsion.

Still other non-CT-CRM adjuvants include mineral oil and water emulsions, aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic polyols, muramyl dipeptide, killed Bordetella, saponins, such as Stimulon.TM. QS-21 (Antigenics, Framingham, Mass.), described in U.S. Pat. No. 5,057,540, which is hereby incorporated by reference, and particles generated therefrom such as ISCOMS (immunostimulating complexes), Mycobacterium tuberculosis, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Pat. No. 6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129; see, e.g., International Patent Publication Nos. WO 93/13302 and WO 92/19265, incorporated herein by reference.

Various cytokines and lymphokines are also suitable for inclusion in the immunogenic compositions of this invention. One such cytokine is granulocyte-macrophage colony stimulating factor (GM-CSF), which has a nucleotide sequence as described in U.S. Pat. No. 5,078,996, which is hereby incorporated by reference. A plasmid containing GM-CSF cDNA has been transformed into E. coli and has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209, under Accession Number 39900. The cytokine Interleukin-12 (IL-12) is another adjuvant that is described in U.S. Pat. No. 5,723,127, which is hereby incorporated by reference (available from Genetics Institute, Inc., Cambridge, Mass.). Other cytokines or lymphokines have been shown to have immune modulating activity, including, but not limited to, the interleukins 1-.alpha., 1-.beta., 2, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 17 and 18, the interferons-.alpha., .beta. and .gamma., granulocyte colony stimulating factor, and the tumor necrosis factors .alpha. and .beta., and are suitable for use as adjuvants.

Still other suitable optional components of the immunogenic compositions of this invention include, but are not limited to: surface active substances (e.g., hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyl-dioctadecylammonium bromide), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextransulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin; oil emulsions; and mineral gels, e.g., aluminum phosphate, etc. and immune stimulating complexes. The CT-CRM and antigen may also be incorporated into liposomes, or conjugated to polysaccharides, lipopolysaccharides and/or other polymers for use in an immunogenic composition.

Immunogenic compositions of this invention including the CT-CRM mutant(s), or DNA sequences and molecules encoding the desired CT-CRM of this invention, are also useful as polynucleotide compositions (also known as DNA immunogenic compositions) or administered with polynucleotides encoding the selected antigen. For example, it has been previously demonstrated that BALB/c mice administered a formulation of plasmid DNA (pDNA) encoding the full length glycoprotein D of herpes simplex virus (HSV) type 2 (gD2), along with CT-CRM.sub.E29H by the intradermal route generated a higher average cellular response than those that received plasmid DNA encoding HSV gD2 by itself by the intradermal route. In addition, the average serum antibody titers for mice, which, received the plasmid DNA HSV gD2 composition along with CT-CRM.sub.E29H was approximately the same as that seen in mice that received the plasmid DNA HSV gD2 composition without adjuvant. Similarly, the plasmid DNA HSV gD2 composition adjuvanted with CT-CRM.sub.E29H also generated a gD2-specific antibody response in vaginal wash samples at levels that were comparable to those seen following the delivery of the non-adjuvanted composition by intradermal or intramuscular routes. Mice immunized with the plasmid DNA HSV gD2 composition adjuvanted with CT-CRM.sub.E29H or CT and delivered by the intradermal route also generated substantially higher levels of gamma interferon and IL-5 than mice that received the plasmid DNA HSV-gD2 composition without adjuvant. Thus, CT-CRMs enhance proliferative and gamma interferon responses when administered with a plasmid DNA composition against HSV.

In addition to a carrier as described above, immunogenic compositions composed of polynucleotide molecules desirably contain optional polynucleotide facilitating agents or "co-agents", such as a local anesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation such as polylysine, a branched, three-dimensional polycation such as a dendrimer, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells. Such a facilitating agent includes bupivicaine (see U.S. Pat. No. 5,593,972, which is hereby incorporated by reference). Other non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U.S. Pat. Nos. 5,703,055; 5,739,118; 5,837,533; International Patent Publication No. WO96/10038, published Apr. 4, 1996; and International Patent Publication No WO94/16737, published Aug. 8, 1994, which are each incorporated herein by reference.

Most preferably, the local anesthetic is present in an amount that forms one or more complexes with the nucleic acid molecules. When the local anesthetic is mixed with nucleic acid molecules or plasmids of this invention, it forms a variety of small complexes or particles that pack the DNA and are homogeneous. Thus, in one embodiment of the immunogenic compositions of this invention, the complexes are formed by mixing the local anesthetic and at least one plasmid of this invention. Any single complex resulting from this mixture may contain a variety of combinations of the different plasmids. Alternatively, in another embodiment of the compositions of this invention, the local anesthetic may be pre-mixed with each plasmid separately, and then the separate mixtures combined in a single composition to ensure the desired ratio of the plasmids is present in a single immunogenic composition, if all plasmids are to be administered in a single bolus administration. Alternatively, the local anesthetic and each plasmid may be mixed separately and administered separately to obtain the desired ratio. Where, hereafter, the term "complex" or "one or more complexes" or "complexes" is used to define this embodiment of the immunogenic composition, it is understood that the term encompasses one or more complexes with each complex containing a mixture of the CT-CRM-encoding plasmids and antigen-encoding plasmids, or a mixture of complexes formed discretely, wherein each complex contains only one type of plasmid, or a one or a mixture of complexes wherein each complex contains a polycistronic DNA Preferably, the complexes are between about 50 to about 150 nm in diameter. When the facilitating agent used is a local anesthetic, preferably bupivacaine, an amount of from about 0.1 weight percent to about 1.0 weight percent based on the total weight of the polynucleotide composition is preferred. See, also, International Patent Publication No. WO99/21591, which is hereby incorporated by reference, and which teaches the incorporation of benzylammonium surfactants as co-agents, preferably administered in an amount of between about 0.001-0.03 weight %. According to the present invention, the amount of local anesthetic is present in a ratio to said nucleic acid molecules of 0.01-2.5%

w/v local anesthetic to 1-10 .mu.g/ml nucleic acid. Another such range is 0.05-1.25% w/v local anesthetic to 100 .mu.g/ml to 1 ml/ml nucleic acid.

As used, such a polynucleotide immunogenic composition expresses the CT-CRM and antigens on a transient basis in vivo; no genetic material is inserted or integrated into the chromosomes of the host. This use is thus distinguished from gene therapy, where the goal is to insert or integrate the genetic material of interest into the chromosome. An assay is used to confirm that the polynucleotides administered by immunization do not rise to a transformed phenotype in the host (U.S. Pat. No. 6,168,918).

The immunogenic compositions may also contain other additives suitable for the selected mode of administration of the composition. The composition of the invention may also involve lyophilized polynucleotides, which can be used with other pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19.sup.th edition (1995), e.g., Chapter 95 Aerosols; and International Patent Publication No. WO99/45966, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.

These nucleic acid molecule-containing immunogenic compositions can contain additives suitable for administration via any conventional route of administration. In some preferred embodiments, the immunogenic composition of the invention is prepared for administration to human subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric-coated tablets or capsules, or suppositories.

The immunogenic compositions of the present invention (whether protein-containing or nucleic acid molecule-containing compositions), as described above, are not limited by the selection of the conventional, physiologically acceptable, carriers, adjuvants, or other ingredients useful in pharmaceutical preparations of the types described above. The preparation of these pharmaceutically acceptable compositions, from the above-described components, having appropriate pH isotonicity, stability and other conventional characteristics is within the skill of the art.

E. Methods of Use of the Compositions of this Invention

The immunogenic compositions of this invention that comprise the CT-CRM alone or a combination of the CT-CRM and a selected antigen, are administered to a human or to a non-human vertebrate by a variety of routes to enhance the immune response to an antigen, preferably a disease-causing antigen, as identified above. The compositions of the present invention modulate the immune response by improving the vertebrate host's antibody response and cell-mediated immunity after administration of a composition comprising a selected antigen as described above, and an effective adjuvanting amount of a mutant CT-CRM, where the mutant CT-CRM has substantially reduced toxicity compared to a wild-type CT, and wherein the reduced toxicity is a result of a single amino acid substitution, a single amino acid insertion, a double amino acid insertion or a single amino acid substitution and double amino acid insertion.

In one embodiment, the immunogenic composition containing the CT-CRM (either as a protein or encoded by a nucleic acid molecule) is administered prior to administration of a composition comprising the selected antigen (either as a protein or as a nucleic acid). In another embodiment, the immunogenic composition is administered simultaneously with the antigen, whether it is administered in a composition containing both antigen and CT-CRM or as a separate composition from that of the antigen-containing composition. In still a further embodiment, the composition containing the CT-CRM is administered after the composition containing the antigen. It is preferable, although not required, that the antigen and the mutant CT-CRM be administered at the same time.

The immunogenic composition containing the CT-CRM may be administered as a protein or as a nucleic acid molecule encoding the protein, as described above. The immunogenic composition containing the CT-CRM may be administered as a protein in combination with a selected antigen administered as a protein. Alternatively, as described above, the CT-CRM immunogenic composition may be administered as a protein with a nucleic acid molecule encoding the antigen, as described above. Still another alternative involves administering both the CT-CRM and the antigen as nucleic acid sequences encoding these proteins.

Any suitable route of administration may be employed to administer the immunogenic composition containing the CT-CRM. The route may be the same or different from a route selected to administer a composition containing the selected antigen, if the CT-CRM and antigen are administered in separate compositions or in different forms, e.g., protein or nucleic acids. Suitable routes of administration include, but are not limited to, intranasal, oral, vaginal, rectal, parenteral, intradermal, transdermal (see, e.g., International patent publication No. WO 98/20734, which is hereby incorporated by reference), intramuscular, intraperitoneal, subcutaneous, intravenous and intraarterial. The appropriate route is selected depending on the nature of the immunogenic composition used, and an evaluation of the age, weight, sex and general health of the patient and the antigens present in the immunogenic composition, and similar factors by an attending physician.

In general, selection of the appropriate "effective amount" or dosage for the the CT-CRM and/or antigen components of the immunogenic composition(s) of the present invention will also be based upon the protein or nucleic acid form of the CT-CRM and antigen, the identity of the antigen in the immunogenic composition(s) employed, as well as the physical condition of the subject, most especially including the general health, age and weight of the immunized subject. The method and routes of administration and the presence of additional components in the immunogenic compositions may also affect the dosages and amounts of the CT-CRM and antigen. Such selection and upward or downward adjustment of the effective dose is within the skill of the art. The amount of CT-CRM and antigen required to induce an immune response, preferably a protective response, or produce an exogenous effect in the patient without significant adverse side effects varies depending upon these factors. Suitable doses are readily determined by persons skilled in the art.

As an example, in one embodiment, for the compositions containing protein components, e.g., a CT-CRM variant protein and/or antigen as described above, each dose may comprise between about 1 .mu.g to about 20 mg of the protein per mL of a sterile solution. Other dosage ranges may also be contemplated by one of skill in the art. Initial doses may be optionally followed by repeated boosts, where desirable.

In another example, the amounts of nucleotide molecules in the DNA and vector compositions may be selected and adjusted by one of skill in the art. In one embodiment, each dose will comprise between about 50 .mu.g to about 1 mg of CT-CRM-encoding or antigen-encoding nucleic acid, e.g., DNA plasmid, per mL of a sterile solution.

The number of doses and the dosage regimen for the composition are also readily determined by persons skilled in the art. Protection may be conferred by a single dose of the immunogenic composition containing the CT-CRM, or may require the administration of several doses with or without the selected antigen, in addition to booster doses at later times to maintain protection. In some instances, the adjuvant property of the mutant CT-CRM may reduce the number of doses containing antigen that are needed or may reduce the time course of the dosage regimen. The levels of immunity can be monitored to determine the need, if any, for boosters.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

All references cited herein are hereby incorporated by reference.

EXAMPLE 1

Expression of CT Mutants

A. Bacterial Strains, Plasmids and Growth Conditions.

E. coli TG1 (Amersham Corporation, Arlington Heights, Ill.), TX1, a naladixic-acid resistant derivative of TG1 carrying F'Tc, lacI.sup.q from XL1blue (Stratagene, La Jolla, Calif.), TE1 (TG1 ends, F'Tc, lacI.sup.q) and CJ236(F'Tc, lacI.sup.q) (BioRad, Hercules, Calif.) were used as hosts for cloning recombinant plasmids and expression of variant proteins. Plasmid-containing strains were maintained on LB agar plates with antibiotics as required (ampicillin, 50 .mu.g/ml; kanamycin 25 .mu.g/ml; tetracycline 10 .mu.g/ml).

B. Mutagenesis of ctxA Gene.

Site-directed mutagenesis using single-stranded uracil-containing templates (Jobling, M. G. and Holmes, R. K., 1992 Infect. Immun., 60:4915-24) was used to select for oligonucleotide-derived mutants created in plasmid pMGJ67, a clone of the native CT operon in pSKII- (Stratagene). Briefly, each oligonucleotide was phosphorylated and used to direct second strand synthesis on a single-stranded DNA template rescued from dut ung CJ236 (F'Tc, pMGJ67). Following ligation and transformation of ung.sup.+ strain TX1, single-stranded DNA was rescued from Ap.sup.R transformants and sequenced by the dideoxy chain termination method (Kunkel, T. A., 1985 Proc. Natl. Acad., Sci., USA, 82:488-492). Some mutations were introduced directly into pARCT2 using the QuickChange mutagenesis method (Stratagene). pARCT2 is an arabinose-inducible clone derived from pAR3 (International Patent Publication No. WO98/20734) expressing an operon containing the ctxA and ctxB genes with signal sequences derived from the LTIIb B gene, and with each gene independently using the translation inititation sequences derived from T7 gene 10 from vector plasmid pT7-7, a derivative of pT7-1.

C. One and Two Codon Insertion Mutations.

Single codon insertions were generated at DdeI restriction sites by partial digestion of pMGJ64 (a derivative of pMGJ67), followed by filling-in of the 3-base sticky ends and self-ligation. Two codon TAB-linker insertion mutations were made by adding six base-pair ApaI linkers (GGGCCC) to the ends of RsaI partial digests of pMGJ64 as described in the TAB manual (Pharmacia). Transformants were screened for loss of either a single DdeI or RsaI site (and presence of a new ApaI site) and confirmed by DNA sequencing.

D. Construction of Arabinose Promoted CT-CRM Expression Vectors.

Previous experience with CT-CRM.sub.E29H (International Patent Publication No. WO 00/18434) has shown that maximal production in E. coli could be achieved by substituting synthetic Shine-Delgaro sequences upstream of the ctxA gene and placing the operon under the control of the arabinose promoter system. CT operons containing site directed mutations in the A subunit were made as previously described (supra). CT-CRMs were originally under the control of a .beta.-galactosidase promoter and expression levels in E. coli were low. PCR was used to modify the region 5' to the ATG of the CT-A subunit and insert an NheI site at the 5' end. The corresponding 3' primer added a HindIII site at the 3' end of the CT-B gene. Primer sequences used were:

TABLE-US-00002 CT29FNhe: 5' TTTTTTGGGCTAGCATGGAGGAAAAGATGAGC (SEQ ID NO: 5) CT29RHnd: 5' CGAGGTCGAAGCTTGCATGTTTGGGC. (SEQ ID NO: 6)

PCR was performed on each mutant CT-CRM operon and the PCR products were ligated into pCR2.1-Topo (Invitrogen) according to the manufacturer's directions and transformed into Top10F' cells. Recombinant E. coli were plated onto SOB agar containing Kanamycin (25 .mu.g/ml) and X-gal (40 .mu.g/ml). Plasmids from white colonies were screened for inserts by digestion with EcoRI. Plasmids containing inserts of the correct size were digested with NheI and HindIII according to the manufacturer's directions and the DNA fragments containing the CT operons isolated from low melting point agarose. Plasmid pBAD 18-Cm (Invitrogen) was digested with NheI-HindIII and the linear DNA isolated from low melting point agarose. Digested pBAD18 and the CT operons were ligated at 12.degree. C. and transformed into Top10F E coli. Plasmids from chloramphenicol-resistant colonies were screened for inserts by restriction analysis, and representative clones were sequenced to confirm the presence of the site directed mutations. Plasmids were transformed into DH5.alpha. for expression of CT-CRMs.

E. Expression of CT-CRMs in E. coli.

E. coli DH5.alpha. cells containing plasmids pLP9911, pLP915, pLP907, pLP909 and pLP910, cells expressing the CT-CRMs respectively, were grown m phosphate buffered Hy-Soy media containing chloramphenicol (25 .mu.g/ml) and glycerol (0.5%) at 37.degree. C. with aeration. When cultures reached an OD.sub.600 of approximately 4.5-5.5, they were induced by addition of L-arabinose to a final concentration of 0.5%. Cultures were incubated at 37.degree. C. with aeration for three hours post-induction and then the cells collected by centrifugation. Cell pellets were stored at -20.degree. C.

F Preparation and Purification of CT-CRMs.

Cell pellets were thawed at room temperature and resuspended in 10 mM NaPO.sub.4 and 1 mM EDTA (pH 7.0) at 9% of the original culture volume. Cell suspensions were mechanically disrupted in a microfluidizer and centrifuged for 10 minutes at 8,500.times.g. Cell lysates were further clarified at 160,000.times.g for one hour. The clarified cell lysate was loaded, at a flow rate of 2 ml/minute, onto a carboxymethyl (CM)-sepharose.TM. column (300 ml CM-Sepharose.TM. per 10 liters of culture) (Amersham, Pharmacia) equilibrated with 10 mM NaPO.sub.4 (pH 7.0). The column was washed with >10 volumes of 10 mM NaPO.sub.4 (pH 7.0) at a flow rate of 5 ml/minute. CT-CRM.sub.E29H holotoxin was eluted with four column volumes of 10 mM NaPO.sub.4 (pH 8.3). Purified CT-CRMs were buffer exchanged by dialysis into PBS and stored at 4.degree. C. The presence of intact holotoxin and the respective subunits was determined by native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, respectively. Native PAGE indicated the presence of a purified molecule of 86 kDa (data not shown), the expected molecular weight for intact cholera holotoxin (Tebbey et al, 2000 Vaccine, 18(24):2723-2734). In addition, SDS-PAGE showed two bands that aligned with the CT-A (27 kDa) and CT-B (12 kDa) subunits that comprise the intact holotoxin (data not shown).

EXAMPLE 2

Non-Denaturing Polyacrylamide Gel Electrophoresis

Mutant CT-CRMS, CT-CRM.sub.R25W, CT-CRM.sub.R25G, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP and CT-CRM.sub.Y30WAH, were analyzed by non-denaturing page electrophoresis to determine the percentage of the CT-CRMs present after purification as intact holotoxin. Purified CT-CRMs, 15 .mu.l each (at various protein concentrations), were run through a 6% polymerized non-denaturing polyacrylamide gel. Three different concentrations (300, 600 and 1200 ng) of CT-B were used as a standard. After electrophoresis the gel was stained with Coomassie blue. The gel was then scanned using a densitometer, and the percentage of the holotoxin was calculated from the densitometer readings of the CT-CRMs and CT-B standard. The data indicated that 95% of CT-CRM.sub.R25W, 91.20% of CT-CRM.sub.R25G, 91.00% of CT-CRM.sub.T48TH, 98.80% of CT-CRM.sub.G34GGP and 90.93% of CT-CRM.sub.Y30WAH were present as intact holotoxins (Table 2).

TABLE-US-00003 TABLE 2 Native Gel Assay for Intact Holotoxin CT-CRM % of holotoxin CT-CRM.sub.R25W >95 CT-CRM.sub.R25G 91.0 CT-CRM.sub.T48TH 91.20 CT-CRM.sub.G34GGP 98.80 CT-CRM.sub.Y30WAH 90.93

EXAMPLE 3

Y-1 Adrenal Cell Assay for Residual Toxicity of CT-CRMs

Mutant CT-CRMs were compared with wild-type CT for toxicity in the mouse Y-1 adrenal tumor cell assay, which is used in vitro to measure toxicity of enterotoxins in the cholera toxin/heat labile enterotoxin family. The assay depends upon binding of the toxin to cell surface receptors, and the subsequent entry of the A1 subunit of the toxin into the cytoplasm of the cell.

Native cholera toxin isolated from V. cholerae is proteolytically nicked at the A1-A2 junction, resulting in the A1 and A2 subunits being held together by only a disulfide bond. This makes the A1 and A2 subunits unstable and easily dissociable from each other. The A1 subunit of the nicked CT dissociates from the A2 subunit upon binding to the cell surface receptor, and enters the cell, where it ADP-ribosylates the regulatory G-protein (Gs.alpha.), leading to its toxic effects as described above. In contrast, enterotoxin produced in E. coli (either CT or LT) is unnicked, and thus, has the A1-A2 peptides still joined. Consequently, the CT produced in V. cholerae is significantly more toxic in the Y-1 adrenal cell assays than the CT produced in a heterologous bacterial cell such as E. coli.

In a first Y-1 adrenal cell assay, mutant CT-CRMs were compared to nicked wild-type CT from V. cholerae for toxicity. In this assay, Y-1 adrenal cells (ATCC CCL-79) were seeded in 96-well flat-bottom plates at a concentration of 10.sup.4 cells per well. Thereafter, three-fold serial dilutions of purified (.about.90% purity as determined by Coomassie staining) CT-CRMs were added to the tumor cells and incubated at 37.degree. C. (5% CO.sub.2) for 18 hours. The cells were then examined by light microscopy for evidence of toxicity (cell rounding). The endpoint titer was defined as the minimum concentration of toxin required to give greater than 50% cell rounding. The percent of residual toxicity was then calculated using the endpoint titer of wild-type nicked CT from V. cholerae (100% toxicity) divided by the titer elicited by CT-CRMs multiplied by 100. The data set forth in Table 3 indicate that the residual toxicity of the five purified mutant holotoxins, CT-CRM.sub.R25W, CT-CRM.sub.R25G, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP and CT-CRM.sub.Y30WAH tested using the Y-1 adrenal cell assay was substantially reduced.

TABLE-US-00004 TABLE 3 Y-1 Adrenal Cell Assay CT-CRM % Residual Toxicity CT-CRM.sub.R25W 0.37 CT-CRM.sub.R25G 0.041 CT-CRM.sub.T48TH 0.12 CT-CRM.sub.G34GGP 1.11 CT-CRM.sub.Y30WAH 0.12

In a second independent study, crude periplasmic extracts of E. coli cells (TG1) expressing elevated levels of mutant CT-CRMs, were compared against unnicked wild-type CT holotoxin expressed in E. coli for residual toxicity in Y-1 adrenal cell assay. Y-1 adrenal cells were incubated in multi-well dishes in an RPMI medium containing 10% fetal calf serum in the presence of crude E. coli cell lysate. Cell toxicity was monitored as before. In this study, one toxic unit was defined as the smallest amount of toxin or supernatant that caused rounding of 75-100% of the cells in a well after overnight incubation. The results of this study are presented in Table 4 below.

TABLE-US-00005 TABLE 4 Y-1 Adrenal Cell Assay CT-CRM % Residual Toxicity CT-CRM.sub.R25W 30 CT-CRM.sub.R25G 6 CT-CRM.sub.T48TH 25 CT-CRM.sub.G34GGP 30 CT-CRM.sub.Y30WAH 8

The results of this study indicated that while the toxicities of CT-CRM.sub.R25G and CT-CRM.sub.Y30WAH were substantially reduced, the toxicities of CT-CRM.sub.R25W and CT-CRM.sub.T48TH were approximately 30% of the toxicity of wild-type CT. Without being bound by theory, the variant results in the second study (Table 4) may be attributable to the fact that periplasmic crude E. coli cell lysates used in the second study contained unnicked mutant CT-CRMs. Another contributing factor may be that toxicity was measured as a percentage of the toxicity of wild-type, unnicked CT produced by E. coli, wherein the unnicked wild-type CT from E. coli had a 50% cell rounding dose of 6250 pg/ml in the same Y1 cell assay (data not shown). In contrast, in the first study, the residual cytotoxicity of the mutant CT-CRMs is expressed as a percentage of the toxicity of wild-type, nicked CT produced by V. cholerae, wherein the nicked holotoxin had a 50% cell rounding dose of 125 pg/ml in the same Y1 cell assay. Consequently, the residual toxicity reported in the second study is 50 fold higher than that obtained in the first study.

EXAMPLE 4

The ADP-Ribosyltransferase Assay

NAD.sup.+:agmatine ADP-ribosyltransferase activity was measured as the release of [carbonyl-.sup.14C] nicotinamide from radiolabeled NAD.sup.+. Briefly, CT and CT-CRMs were trypsin activated and incubated for 30 minutes at 30.degree. C. with 50 mM glycine/20 mM dithiothreitol in TEAN buffer (Tris/EDTA/sodium azide/sodium chloride) (pH 8.0). Thereafter, the following materials were added to the reaction: 0.1 .mu.g of soybean trypsin inhibitor, 50 mM potassium phosphate, 10 mM agmatine, 20 mM dithiothreitol, 10 mM magnesium chloride, 100 .mu.M GTP, 3 mM dimyristoylphosphatidyl-choline, 0.2% cholate, 0.03 mg of ovalbumin, 100 .mu.M [adenine-U-.sup.14C]NAD (DuPont NEN.TM., Boston, Mass.) and water to a final volume of 300 .mu.l. After incubation for 90 minutes at 30.degree. C., 100 .mu.l samples were applied to columns (0.64.times.5 cm) of AG1-X2 (Bio-Rad), which were washed five times with 1.0 ml of distilled/deionized H.sub.2O. Eluates containing [.sup.14C]ADP-ribosylagmatine were collected for radioassay. Mean recovery of .sup.14C in the eluate is expressed as percentage of that applied to column. The results are presented in Table 5.

TABLE-US-00006 TABLE 5 NAD:Agmatine ADP-Ribosyltransferase Activity ADP-ribosylagmatine % ADP- formed ribosylation CT/CT-CRM (nmol/hr/.mu.g protein) activity CT, 10 .mu.g 35.7 100 CT-CRM.sub.R25W 1.6 4.5 CT-CRM.sub.R25G 1.0 2.7 CT-CRM.sub.T48TH 1.2 3.4 CT-CRM.sub.G34GGP 1.8 5.0 CT-CRM.sub.Y30WAH 1.6 4.5

ADP-ribosyltransferase activity was also independently determined using diethylamino (benzylidine-amino) guanidine (DEABAG) as a substrate (Jobling, MG and Holmes, R K 2001 J. Bacteriol., 183(13):4024-32). In this assay, 25 .mu.l aliquots of mutant CT-CRMs from purified cell lysates, activated for 30 minutes at 30.degree. C. with 1/50 w/w trypsin, were incubated with 200 .mu.l 2 mM DEABAG in 0.1M K.sub.2PO.sub.4, pH 7.5, 10 .mu.M NAD, 4 mM DTT for two

hours. The reaction was stopped by adding 800 .mu.l of a slurry buffer containing 400 mg DOWEX AG50-X8 resin to bind unreacted substrate. ADP-ribosylated DEABAG in the supernatant was quantitated by florescence emission in a DyNA Quant fluorimeter calibrated with DEABAG. With the exception of the mutants CT-CRM.sub.G34GGP and CT-CRM.sub.Y30WAH, the ADP ribosyl-transferase activities of the mutant CT-CRMs were substantially reduced over that of wild-type (Table 6). The high level of ADP-ribosyl-transferase activity seen with CT-CRM.sub.G34GGP and CT-CRM.sub.Y30WAH may be attributable to the fact that in this study the ADP ribosyl-transferase activity of mutant CT-CRMs was measured using a different substrate in a different assay protocol.

TABLE-US-00007 TABLE 6 ADP-ribosyltransferase Activity of CT-CRMs using Diethylamino (benzylidine-amino) Guanidine (DEABAG) CT/CT-CRM % ADP-ribosylation Activity CT 100 CT-CRM.sub.R25W 10 CT-CRM.sub.R25G 0.5 CT-CRM.sub.T48TH 18 CT-CRM.sub.G34GGP 54 CT-CRM.sub.Y30WAH 43

EXAMPLE 5

Immune Responses of BALB/C Mice Immunized with Recombinant P4 Outer Membrane Protein (rP4) of Nontypable Haemophilus influenzae (NTHi) Alone or in Conjunction with CT-CRMS

BALB/c mice (6-8 weeks old, 5 mice/group) were immunized at weeks 0, 3 and 5 with recombinant P4 protein (rP4, 5 .mu.g per dose) in saline or co-formulated with wild-type CT, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W or CT-CRM.sub.R25G at a dose 1.0 .mu.g per immunization. A total volume of 10 .mu.l was administered intranasally (5 .mu.l per nostril). Mice were bled at weeks 0, 2, 3, 4, 5 or 6 in order to assay serum antibody responses. One week after the last immunization (week 6), mice were sacrificed for the analysis of mucosal antibody responses.

Significant differences between groups were determined by the Tukey-Kramer HSD multiple comparisons test (for rP4 protein) or by the Student t-test (for UspA2) using JMP.RTM. statistical discovery software (SAS Institute Inc., Cary, N.C.).

Analysis of serum antibodies at weeks 0, 3, 5 and 6 showed that immunization with NTHi rP4 protein formulated with any of the CT-CRM mutants, disclosed herein at a concentration of 1 .mu.g/dose, significantly induced immune responses to rP4 protein. The magnitude of the total IgG immune response to rP4 protein was increased approximately 15-35 fold by inclusion of the CT-CRM mutants in the formulation. No significant differences were observed in total anti-rP4 IgG titers among the new mutant toxins (CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W and CT-CRM.sub.R25G) even though they all elicited significantly higher IgG titers than rP4 protein alone by Student t-test (Table 6). Individual serum analysis of IgA antibodies showed that only formulation rP4/CT-CRM.sub.R25G elicited significantly higher titers of IgA antibodies to rP4 protein than the control group receiving rP4 protein in saline (Table 7). The use of each of the new CT-CRM mutants also enhanced serum IgG subclass antibodies (IgG1, IgG2a and IgG2b) to rP4 protein (Table 9).

Anti-rP4 protein antibody responses were also analyzed in pooled mucosal wash samples (Table 8). As expected, no induction of antibody in BAL and NW from rP4/saline immunized mice was observed. However, the potent mucosal adjuvant capacity of CT-CRM.sub.T48TH, CT-CRM.sub.Y30WAH and CT-CRM.sub.R25G was clearly demonstrable. Although no statistical analysis can be performed on these pooled samples, some trends appeared. For example, mice that received CT-CRM.sub.T48TH, CT-CRM.sub.Y30WAH and CT-CRM.sub.R25G displayed elevated rP4 specific IgA antibodies in each of the saliva, NW and VW samples tested.

Additionally, anti-CT antibody responses were also determined. As shown in Tables 7-11, all the CT mutants enhanced the systemic and mucosal CT-specific antibody responses one-week after the last immunization. However, it should be noted that the mice in the above study were, subsequent to the completion of the study, determined to be infected with the mouse hepatitis virus.

TABLE-US-00008 TABLE 7 The Effect of Mutant Cholera Toxins on the Immunogenicity of NTHi LrP4 Protein following Intranasal Immunization in BALB/c Mice. Anti-rLP4 Antibody Titers.sup.c (Pooled Sera).sup.d Week 0 Week 3 Week 5 Week 6 Immunogen Rte.sup.a Adjuvant.sup.b IGA.sup.b IgG IgA IgG IgA IgG IgA IgG NTHi LrP4 IN -- <100 <100 <100 168 <100 532 122 3,638 NTHi LrP4 IN CT <100 120 <100 795 198 18,584 NTHi LrP4 IN CT-CRM.sub.E29H <100 <100 <100 576 152 4,854 NTHi LrP4 IN CT-CRM.sub.T48TH <100 102 111 10,594 453 55,775 NTHi LrP4 IN CT-CRM.sub.G34GGP <100 146 196 1,701 434 68.325 NTHi LrP4 IN CT-CRM.sub.Y30WAH <100 <100 <100 3,406 391 93,502 NTHi LrP4 IN CT-CRM.sub.R25W <100 187 116 16,809 509 127,130 NTHi LrP4 IN CT-CRM.sub.R25G <100 278 273 23,163 1,056 62,323 .sup.aFemale BALB/c mice were immunized with NTHi rP4 (5 .mu.g) at weeks 0, 3, and 5. IN vax = 10 .mu.l .sup.bNTHi rP4 immunogens were formulated with saline or 1 .mu.g each of Cholera Toxin, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT-CRM.sub.R25G .sup.cELISAs were performed using 0.2 .mu.g NTHI rP4 per well and with an endpoint titer determination of 0.1 at OD.sub.405. .sup.dSera samples were collected at weeks 0, 3, 5 and 6; pooled samples represent an n of 5.

TABLE-US-00009 TABLE 8 The Effect of Mutant Cholera Toxins on the Immunogenicity of NTHi LrP4 Protein following Intranasal Immunization in BALB/c Mice. Anti-rLP4 Antibody Titers.sup.c (Pooled Mucosal Washes).sup.d SAL BAL VW NW Immunogen Rte.sup.a Adjuvant.sup.b IgA IgG IgA IgG IgA IgG IgA IgG NTHi LrP4 IN -- 19 <10 <10 <10 33 <10 <10 <10 NTHi LrP4 IN CT <10 <10 <10 23 <10 <10 <10 <10 NTHi LrP4 IN CT-CRM.sub.E29H <10 <10 <10 <10 33 <10 <10 - <10 NTHi LrP4 IN CT-CRM.sub.T48TH 105 <10 <10 79 73 21 12 <10 NTHi LrP4 IN CT-CRM.sub.G34GGP 17 <10 <10 80 11 23 <10 13 NTHi LrP4 IN CT-CRM.sub.Y30WAH 25 <10 <10 113 48 47 10 19 NTHi LrP4 IN CT-CRM.sub.R25W 37 <10 <10 169 20 23 <10 <10 NTHi LrP4 IN CT-CRM.sub.R25G 185 <10 <10 64 348 32 23 <10 .sup.aFemale BALB/c mice were immunized with NTHi rP4 (5 .mu.g) at weeks 0, 3, and 5. IN vax = 10 .mu.l .sup.bNTHi rP4 immunogens were formulated with saline or 1 .mu.g each of Cholera Toxin, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT- CRM.sub.R25G .sup.cELISAs were performed using 0.2 .mu.g NTHI rP4 per well and with an endpoint titer determination of 0.1 at OD.sub.405. .sup.dMucosal samples were collected at week 6, day 1; pooled samples represent an n of 5.

TABLE-US-00010 TABLE 9 The Effect of Mutant Cholera Toxins on the Immunogenicity of NTHi LrP4 Protein following Intranasal Immunization in BALB/c Mice. Anti-rLP4 Antibody Titers.sup.c (Week 6 Pooled Sera).sup.d Immunogen Rte.sup.a Adjuvant.sup.b IgG1 IgG2a IgG2b IgG3 NTHi LrP4 IN -- 1,165 1,891 1,245 <100 NTHi LrP4 IN CT 625 14,989 9,284 278 NTHi LrP4 IN CT- 1,630 2,618 845 <100 CRM.sub.E29H NTHi LrP4 IN CT- 11,220 35,239 20,733 206 CRM.sub.T48TH NTHi LrP4 IN CT- 12,583 48,134 24,267 <100 CRM.sub.G34GGP NTHi LrP4 IN CT- 13,894 59,049 28,975 744 CRM.sub.Y30WAH NTHi LrP4 IN CT- 24,373 89,892 37,389 422 CRM.sub.R25W NTHi LrP4 IN CT- 7,957 46,776 16,731 256 CRM.sub.R25G .sup.aFemale BALB/c mice were immunized with NTHi rP4 (5 .mu.g) at weeks 0, 3, and 5. IN vax = 10 .mu.l .sup.bNTHi rP4 immunogens were formulated with saline or 1 .mu.g each of Cholera Toxin, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT-CRM.sub.R25G .sup.cELISAs were performed using 0.2 .mu.g NTHI rP4 per well and with an endpoint titer determination of 0.1 at OD.sub.405. .sup.dSera samples were collected at weeks 0, 3, 5 and 6; pooled samples represent an n of 5.

TABLE-US-00011 TABLE 10 The Effect of Mutant Cholera Toxins on the Immunogenicity of NTHi LrP4 Protein following Intranasal Immunization in BALB/c Mice. Anti-rLP4 Antibody Titers on Individual Sera.sup.c,d Immunogen Rte.sup.a Adjuvant.sup.b 1 2 3 4 5 GeoMean.sup.c StDev NTHi LrP4 IN -- 35 114 61 79 316 121 113 NTHi LrP4 IN CT 139 48 145 85 461 176 165 NTHi LrP4 IN CT-CRM.sub.E29H 33 126 333 26 49 113{circumflex over ( )} 129 NTHi LrP4 IN CT-CRM.sub.T48TH 468 780 530 76 218 414 275 NTHi LrP4 IN CT-CRM.sub.G34GGP 177 479 963 175 214 402 338 NTHi LrP4 IN CT-CRM.sub.Y30WAH 271 443 408 699 282 421 173 NTHi LrP4 IN CT-CRM.sub.R25W 431 198 361 360 835 437 238 NTHi LrP4 IN CT-CRM.sub.R25G 1,037 462 1,851 1,825 678 1,171*{circumflex over ( )} 643 .sup.aFemale BALB/c mice were immunized with NTHi rP4 (5 .mu.g) at weeks 0, 3, and 5. IN vax = 10 .mu.l .sup.bNTHi rP4 immunogens were formulated with saline or 1 .mu.g each of Cholera Toxin, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT-CRM.sub.R25G .sup.cELISAs were performed using 0.2 .mu.g NTHI rP4 per well and with an endpoint titer determination of 0.1 at OD.sub.405. .sup.dSera samples were collected at weeks 0, 3, 5 and 6; pooled samples represent an n of 5. .sup.e*denotes significant difference compared to saline control; {circumflex over ( )}denotes significant difference compared to other IN groups.

TABLE-US-00012 TABLE 11 The Effect of Mutant Cholera Toxins on the Immunogenicity of NTHi LrP4 Protein following Intranasal Immunization in BALB/c Mice. Anti-rLP4 Antibody Titers on Individual Sera.sup.c,d Immunogen Rte.sup.a Adjuvant.sup.b 1 2 3 4 5 GeoMean.sup.e StDev NTHi LrP4 IN -- 112 2,393 2,885 4,432 8,471 3,659 3,104 NTHi LrP4 IN CT 22,042 5,499 13,292 10,746 24,920 15,300 8,044 NTHi LrP4 IN CT-CRM.sub.E29H 3,063 16,889 179 204 1,406 4,348{circumflex over ( )} 7,109 NTHi LrP4 IN CT-CRM.sub.T48TH 63,090 52,393 53,912 13,017 38,604 44,203* 1- 9,506 NTHi LrP4 IN CT-CRM.sub.G34GGP 63,924 63,908 73,793 59,169 50,229 62,205* - 8,555 NTHi LrP4 IN CT-CRM.sub.Y30WAH 63,522 65,791 31,934 174,833 130,173 93,251- * 57,915 NTHi LrP4 IN CT-CRM.sub.R25W 138,982 88,085 160,963 74,885 151,979 122,979- * 38,957 NTHi LrP4 IN CT-CRM.sub.R25G 47,114 15,915 154,578 34,780 17,598 54,003* 5- 7,680 .sup.aFemale BALB/c mice were immunized with NTHi rP4 (5 .mu.g) at weeks 0, 3, and 5. IN vax = 10 .mu.l .sup.bNTHi rP4 immunogens were formulated with saline or 1 .mu.g each of Cholera Toxin, CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT-CRM.sub.R25G .sup.cELISAs were performed using 0.2 .mu.g NTHI rP4 per well and with an endpoint titer determination of 0.1 at OD.sub.405. .sup.dSera samples were collected at weeks 0, 3, 5 and 6; pooled samples represent an n of 5. .sup.e*denotes significant difference compared to saline control; {circumflex over ( )}denotes significant difference compared to other IN groups.

EXAMPLE 6

The Immune Responses of BALB/C Mice Immunized with the UspA2 Outer Membrane Protein of M. Catarrhalis

In this study, the capacity of mutant CT-CRMs to augment systemic and mucosal immune responses against the native UspA2 outer membrane protein of M. catarrhalis was examined. BALB/C mice (6-8 weeks old, 5 mice/group) were immunized at weeks 0, 3, and 5. Purified UspA2 (5 .mu.g/dose) alone in 10 .mu.l saline or in a 10 .mu.l formulation containing 0.1 .mu.g/dose of wild-type CT or a mutant CT-CRM (CT-CRM.sub.E29H, CT-CRM.sub.R25W, CT-CRM.sub.R25G, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, or CT-CRM.sub.Y30WAH) was administered to Balb/C mice IN (5 ul/nostril) on week 0, 2 and 4. Analysis of serum antibodies at weeks 0, 2, 4 and 6 showed that immunization with UspA2 formulated with any of the aforementioned CT-CRM mutants except CT-CRM.sub.R25G, at a concentration of 0.1 .mu.g/dose, enhanced antibody responses to UspA2 (Table 12). The magnitude of the total IgG immune response to UspA2 was increased approximately 3-10 fold by inclusion of the CT-derived mutants. CT-CRM.sub.G34GGP, CT-CRM.sub.E29H or wild-type CT elicited significantly higher IgG titers than UspA2/PBS by Student t-test. However, no significant differences were observed in total anti-UspA2 IgG titers between each of the new mutant toxins (CT-CRM.sub.E29H, CT-CRM.sub.T48TH, CT-CRM.sub.G34GGP, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W, or CT-CRM.sub.R25G). The use of each of the CT mutants except CT-CRM.sub.R25W, also enhanced serum IgG1, IgG2a and IgG2b antibodies to UspA2 (Table 12).

Anti-UspA2 antibody responses were also analyzed in pooled mucosal wash samples (Table 13). As expected, no induction of antibody in mucosal washes from UspA2/PBS immunized mice was observed. However, the potent mucosal adjuvant capacity of each mutant CT was clearly demonstrated. Although no statistical analyses were performed on these pooled samples, some trends emerged. For example, mice that received CT-CRM.sub.G34GGP displayed elevated IgG or IgA titers to UspA2 in each of the bronchoalveolar lavage (BAL), nasal wash (NW) and vaginal wash (VW) samples collected (Table 13). In comparison, none of the new mutant toxins appeared to be better than CT-CRM.sub.E29H or wild-type CT in adjuvanting local immune responses to UspA2 protein. Protein-specific IgG and IgA levels in the serum and in mucosal lavages were also examined on day 28. All mutant CTs elicited enhanced serum IgG antibody response (data not shown). The levels of IgG and IgA in bronchial, nasal and vaginal washes were also measured. No IgA was detected in any of the washes, and IgG was detected only in a few washes (Table 13). The mice in this study were, subsequent to the completion of the study, determined to be infected with the mouse hepatitis virus.

TABLE-US-00013 TABLE 12 Sera IgG subclass titers against UspA2 Week 6 * IgG1/G2a Antigen Adjuvant IgG1 IgG2a IgG2b IgG3 Ratio UspA2 None 600 346 320 <100 1.7 UspA2 CT- 3,443 6,655 4,027 <100 0.52 CRM.sub.E29H UspA2 CT- 1,350 809 429 <100 1.6 CRM.sub.T48TH UspA2 CT- 5,918 4,480 3,111 <100 1.32 CRM.sub.G34GGP UspA2 CT- 1,991 1,618 809 <100 1.23 CRM.sub.Y30WAH UspA2 CT- 1,772 1,100 1,095 <100 1.61 CRM.sub.R25W UspA2 CT- 301 221 224 <100 1.36 CRM.sub.R25G UspA2 CT 9,050 18,227 10,195 189 0.5 * BALB/c mice (5/group) were immunized intranasally at weeks 0, 2, 4. Sera and mucosal washes collected at week 6. The antigen dose was 5 .mu.g, and adjuvant dose was 0.1 .mu.g per animal. IgG subclass determined by ELISA on pooled sera.

TABLE-US-00014 TABLE 13 Mucosal IgG & IgA titers against UspA2 * BW NW VW Antigen Adjuvant IgG IgA IgG IgA IgG IgA UspA2 None <10 <10 <10 <10 <10 <10 UspA2 CT-CRM.sub.E29H 16 <10 <10 20 24 506 UspA2 CT-CRM.sub.T48TH <10 <10 <10 <10 <10 58 UspA2 CT-CRM.sub.G34GGP 17 <10 <10 31 24 285 UspA2 CT-CRM.sub.Y30WAH <10 <10 <10 32 <10 208 UspA2 CT-CRM.sub.R25W <10 <10 <10 20 <10 29 UspA2 CT-CRM.sub.R25G <10 <10 <10 <10 <10 <10 UspA2 CT 40 <10 <10 85 111 774 * BALB/c mice (5/group) were immunized intranasally at weeks 0, 2, 4. Sera and mucosal washes collected at week 6. The antigen dose was 5 .mu.g, and adjuvant dose was 0.1 .mu.g per animal. IgG and IgA titers determined by ELISA on pooled sera

EXAMPLE 7

Adjuvanticity of the Mutant Cholera Toxin Holotoxins

To create a comprehensive panel of mutant CT-CRMs with different characteristics of toxicity, functionality and immunogenicity, the above-described CT-CRM mutants were analyzed as mucosal adjuvants, and the toxicity and enzymatic activity profiles of each of the mutants were determined. As summarized in Tables 14 through 18, all mutant CT-CRMs have significantly reduced toxicity and enzyme activity compared to wild-type CT. These genetically detoxified mutant CTs were evaluated for their capacity to adjuvant immune responses to native UspA2 protein from M. catarrhalis.

The experiments were performed as follows: BALB/c mice (6-8 weeks old, 5 mice/group) were immunized at weeks 0, 2 and 4 with 5 .mu.g of purified native UspA2 protein in PBS or co-formulated with doses of 0.1 or 1.0 .mu.g per immunization of wild-type CT, or CT-CRM.sub.E29H, or CT-CRM.sub.T48TH, or

CT-CRM.sub.G34GGP, or CT-CRM.sub.Y30WAH, or CT-CRM.sub.R24W or CT-CRM.sub.R25G. A total volume of 10 .mu.l was administered intranasally (5 .mu.l per nostril). Mice were bled at weeks 0, 2, 4, or 6 in order to assay serum antibody responses. Two weeks after the last immunization (week 6), mice were sacrificed for the analysis of mucosal antibody responses. UspA2 ELISA titers were determined at an endpoint of 0.1 at OD.sub.405. Significant differences between groups were determined by the Tukey-Kramer HSD multiple comparisons test using JMP.RTM. statistical discovery software (SAS Institute Inc., Cary, N.C.).

Adjuvanticity of the CT-CRMs can be summarized as follows. Analysis of serum IgG and IgA antibodies at weeks 2, 4 and 6 showed that immunization with UspA2 protein formulated with any of the CT-CRM mutants, except CT-CRM.sub.R25G, at a concentration of 1 .mu.g/dose, significantly enhanced antibody responses to UspA2 protein (Table 15). The magnitude of the total IgG immune response to UspA2 protein was increased approximately 11-68 fold by inclusion of the CT-derived mutants (excluding CT-CRM.sub.R25G) (Table 16). CT-CRM.sub.T48TH, CT-CRM.sub.Y30WAH, CT-CRM.sub.R25W (at 1 .mu.g dose), and CT-CRM.sub.G34GGP (at both 0.1 .mu.g and 1 .mu.g doses) elicited significantly higher IgG and IgA titers than UspA2/PBS by Tukey-Kramer analysis. However, no significant differences were observed in total anti-UspA2 IgG titers between each of the new mutant toxins excluding CT-CRM.sub.R25G (Table 16). The use of each of the CT mutants except CT-CRM.sub.R25G at a 1 .mu.g dose also enhanced serum IgG1, IgG2a and IgG2b antibodies to UspA2 (Table 17). The ratio of the IgG1 and IgG2a/IgG2b titers was approximately 1.0, indicating a balanced Th1/Th2 type of immune response.

Anti-UspA2 protein antibody responses were also analyzed in pooled mucosal wash samples (Table 18). As expected, no induction of antibody in mucosal washes from UspA2/PBS immunized mice was observed. However, the potent mucosal adjuvant capacity of each mutant CT-CRM, excluding CT-CRM.sub.R25G was clearly demonstrated. There were UspA2 specific mucosal IgA antibodies detected in most of the mucosal samples. Although no statistical analysis can be performed on these pooled samples, some trends appeared. For example, mice that received CT-CRM.sub.G34GGP or CT-CRM.sub.R25W displayed elevated IgG or IgA antibodies to UspA2 protein in each of the bronchoalveolar lavage, the nasal wash and the vaginal wash samples collected, similar to the wild-type CT or CT-CRM.sub.E29H.

These CT-CRMs, except CT-CRM.sub.R25G, are potent mucosal adjuvants for M. catarrhalis UspA2 protein. The serum antibody data showed that all the CT-CRMs except CT-CRM.sub.R25G at 1 .mu.g dose are equally as capable in adjuvanting immune responses to UspA2 protein as is CT-CRM.sub.E29H (Table 16). At 0.1 .mu.g of dose, CT-CRM.sub.G34GGP appeared to be more potent than CT-CRM.sub.E29H at the same dose (Table 16). The mucosal wash data appears to suggest that all of these mutant CT-CRMs except CT-CRM.sub.R25G, retain potent mucosal adjuvant properties (Table 18). Furthermore, they all have significantly lower residual toxicity and enzyme activity than wild-type CT as shown in Table 14. Therefore, these mutant CT-CRMs are additional effective mucosal adjuvants.

TABLE-US-00015 TABLE 14 Characterization of the Mutant Cholera Toxins Y-1 cell ADP-Ribosyl- Homoge- Holotoxin toxicity transferase Mutant CT neity (%) (%) (%) activity (%) CT-CRM.sub.T48TH 100.0 91.0 0.12 3.4 CT-CRM.sub.G34GGP 100.0 98.8 1.11 5.0 CT-CRM.sub.Y30WAH 99.0 90.9 0.12 4.5 CT-CRM.sub.R25W 100.0 >95.0 0.37 4.5 CT-CRM.sub.R25G 99.7 91.2 0.041 2.7

TABLE-US-00016 TABLE 15 Adjuvant Effects of Mutant CT on the Immune Response to UspA2 Delivered IN to Female BALB/c Mice Antigen week 2 week 4 week 6 (5 .mu.g) Adjuvant Dose IgG IgA IgG IgA IgG IgA UspA2 None PBS <100 <100 <100 <100 <500 <50 UspA2 CT-CRM.sub.E29H 1 .mu.g 182 54 3,777 <100 11,305 194 0.1 .mu.g <100 <100 160 <100 544 <50 UspA2 CT-CRM.sub.T48TH 1 .mu.g <100 <100 967 <100 3,542 128 0.1 .mu.g <100 <100 <100 <100 568 <50 UspA2 CT-CRM.sub.G34GGP 1 .mu.g 298 <100 6,170 83 15,498 398 0.1 .mu.g 125 <100 775 <100 2,900 98 UspA2 CT-CRM.sub.Y30WAH 1 .mu.g 206 <100 1,275 <100 3,330 81 0.1 .mu.g <100 <100 <100 <100 <500 <50 UspA2 CT-CRM.sub.R25W 1 .mu.g 304 <100 8,335 <100 16,308 196 0.1 .mu.g <100 <100 214 <100 989 <50 UspA2 CT-CRM.sub.R25G 1 .mu.g <100 <100 232 <100 <1,000 <50- 0.1 .mu.g <100 <100 <100 <100 <500 <50 UspA2 Cholera Toxin 1 .mu.g 191 <100 6,119 <100 13,588 254 0.1 .mu.g 351 <100 5,472 108 20,632 399

BALB/c mice (groups of 5) were immunized IN with a 10 ul volume at weeks 0, 2, & 4. Sera were collected at week 6. The UspA2 ELISA titers were determined at an endpoint of 0.1 at OD.sub.405. The Tukey-Kramer analysis showed the following: The 1 .mu.g dose of each adjuvant is statistically significant from the same adjuvant at 0.1 g dose, except the IgG of CT, CT-CRM.sub.G34GGP and IgA of CT-CRM.sub.R25G. Results in Table 16 reported with an asterisk (*) are statistically significant from the UspA2/PBS group. Results indicated with footnote a (.sup.a) are statistically significantly higher than all 0.1 .mu.g doses (except CT) and the 1 .mu.g dose of CT-CRM.sub.R25G. Results indicated with footnote b (.sup.b) are statistically significantly lower than all 1 .mu.g doses except the 1 .mu.g dose of CT-CRM.sub.T48TH. Results indicated with footnote c (.sup.c) are statistically significantly higher than all 0.1 .mu.g doses (except CT) and the 1 .mu.g dose of CT-CRM.sub.R25G. Results indicated with a footnote d (.sup.d) are statistically significantly lower than all 1 .mu.g doses and also the 0.1 .mu.g dose of CT and CT-CRM.sub.G34GGP.

TABLE-US-00017 TABLE 16 Individual Serum Analysis of IgG and IgA Titers against UspA2 Serum Anti-UspA2 Protein Antibody Titers (Mean Antigen Log.sub.10) (5 .mu.g) Adjuvant Dose IgG IgA UspA2 PBS -- 2.21 .+-. 0.36 <25 UspA2 CT-CRM.sub.E29H 1 .mu.g 4.33 .+-. 0.19* 2.59 .+-. 0.20* 0.1 .mu.g 2.68 .+-. 0.34 1.16 .+-. 0.13 UspA2 CT- 1 .mu.g 3.74 .+-. 0.45* 2.20 .+-. 0.66* CRM.sub.T48TH 0.1 .mu.g 2.51 .+-. 0.56 <25 UspA2 CT- 1 .mu.g 4.53 .+-. 0.11* 2.76 .+-. 0.15* CRM.sub.G34GGP 0.1 .mu.g .sup. 3.95 .+-. 0.20.sup.a* .sup. 2.16 .+-. 0.27*.sup.c UspA2 CT- 1 .mu.g 3.84 .+-. 0.30* 2.03 .+-. 0.34* CRM.sub.Y30WAH 0.1 .mu.g 2.05 .+-. 0.56 <25 UspA2 CT-CRM.sub.R25W 1 .mu.g 4.52 .+-. 0.46* 2.52 .+-. 0.25* 0.1 .mu.g 3.25 .+-. 0.39* 1.28 .+-. 0.26 UspA2 CT-CRM.sub.R25G 1 .mu.g .sup. 2.96 .+-. 0.33.sup.b .sup. 1.53 .+-. 0.35.sup.d 0.1 .mu.g 1.89 .+-. 0.27 1.20 .+-. 0.23 UspA2 Cholera 1 .mu.g 4.61 .+-. 0.15* 2.69 .+-. 0.31* Toxin 0.1 .mu.g 4.44 .+-. 0.24* 2.89 .+-. 0.23*

The data reported in Table 17 was based upon the following experiment. Groups of five female BALB/c mice were immunized intranasally at weeks 0, 2, and 4 with 10 .mu.L containing 5 .mu.g nUspA2 adjuvanted with 1 .mu.g CT (Sigma) or CT mutants. Endpoint antibody titers were determined from sera collected at week 6. Data are presented in Table 17 as the geometric mean (.+-.1 SD) of the reciprocal dilution resulting in an OD.sub.405 of 0.1. Statistical analysis by Tukey-Kramer indicated that results marked with an asterisk (*) were significantly higher than the nUspA2/PBS group.

TABLE-US-00018 TABLE 17 The serum anti-nUspA2 responses of BALB/c mice after intranasal immunization with nUspA2 adjuvanted with mutant CTs Antigen Mean log 10 Antibody Titers (.+-.1SD) Group (5 .mu.g) Adjuvant IgG1 IgG2a IgG2b AG673 nUspA2 PBS <2.00 <2.00 <2.00 AG674 nUspA2 CT-CRM.sub.E29H (1 .mu.g) 3.14 .+-. 0.23* 3.44 .+-. 0.41* 2.92 .+-. 0.20 AG676 nUspA2 CT-CRM.sub.T48TH (1 .mu.g) 2.45 .+-. 0.31 2.85 .+-. 0.52* 2.47 .+-. 0.33 AG678 nUspA2 CT-CRM.sub.G34GGP (1 .mu.g) 3.06 .+-. 0.16* 3.55 .+-. 0.09* 3.00 .+-. 0.02 AG680 nUspA2 CT-CRM.sub.Y30WAH (1 .mu.g) 2.61 .+-. 0.28* 2.77 .+-. 0.23* 2.37 .+-. 0.27 AG682 nUspA2 CT-CRM.sub.R25W (1 .mu.g) 3.29 .+-. 0.40* 3.43 .+-. 0.57* 3.07 .+-. 0.30 AG684 nUspA2 CT-CRM.sub.R25G (1 .mu.g) 2.11 .+-. 0.18 2.05 .+-. 0.09 <2.00 AG686 nUspA2 CT (1 .mu.g) 3.14 .+-. 0.28* 3.39 .+-. 0.27* 3.24 .+-. 0.29

For the data in Table 18, BALB/c mice (5/group) were immunized IN with a 10 .mu.l volume at weeks 0, 2 and 4. Mucosal wash samples were collected at week 6. UspA2 ELISA titers were determined at an endpoint of 0.1 at OD.sub.405.

TABLE-US-00019 TABLE 18 UspA2 ELISA - Mucosal Antibody Titers Bronch Nasal Antigen washes washes Vaginal washes (5 .mu.g) Adjuvant Dose IgG IgA IgG IgA IgG IgA UspA2 -- -- <10 <10 <10 <10 <10 <10 UspA2 CT-CRM.sub.E29H 1 .mu.g 21 <10 <10 17 54 500 0.1 .mu.g <10 <10 <10 <10 <10 <10

UspA2 CT-CRM.sub.T48TH 1 .mu.g <10 <10 <10 <10 <10 293 0.1 .mu.g <10 <10 <10 <10 <10 <10 UspA2 CT-CRM.sub.G34GGP 1 .mu.g 22 <10 <10 12 46 1,103 0.1 .mu.g <10 <10 <10 18 <10 617 UspA2 CT-CRM.sub.Y30WAH 1 .mu.g 11 <10 <10 <10 12 105 0.1 .mu.g <10 <10 <10 <10 <10 <10 UspA2 CT-CRM.sub.R25W 1 .mu.g 24 <10 <10 24 <10 323 0.1 .mu.g <10 <10 <10 <10 <10 13 UspA2 CT-CRM.sub.R25G 1 .mu.g <10 <10 <10 <10 <10 <10 0.1 .mu.g <10 <10 <10 <10 <10 <10 UspA2 Cholera Toxin 1 .mu.g 41 24 <10 14 19 990 0.1 .mu.g 24 10 <10 47 41 460

EXAMPLE 8

The Immune Responses of BALB/c Mice Immunized with the Purified Native Fusion (F) Protein of Respiratory Syncytial Virus (RSV)

The capacity of the mutant CT-CRMs of the present invention to augment the mucosal immune responses against respiratory syncytial virus (RSV) proteins was examined using the purified native fusion (F) protein.

Naive BALB/c mice (8-10 weeks of age, 5/group) were immunized (IN, 10 .mu.l) at weeks 0 and 3 with native purified fusion (F) protein purified from the 248/404 strain of RSV. The protein (3 .mu.g/dose) was prepared in mixture with 1.0 or 0.1 .mu.g of the indicated CT-CRM. Control mice were immunized with F protein admixed with CT-CRM.sub.E29H alone, with wild-type CT, or with PBS. Serum (geometric mean titer.+-.1 standard deviation) and bronchoalveolar (BAW), nasal (NW) and vaginal (VW) wash fluids were collected two weeks after secondary immunization for the determination of end-point anti-F protein total and subclass IgG and IgA titers by ELISA. The mucosal wash samples were pooled for the determination of endpoint titers.

The results from two experiments are presented in Tables 19 and 20.

TABLE-US-00020 TABLE 19 The Humoral Immune Response to BALB/c Mice after Intranasal Immunization with F Protein and CT-CRMs Geometric Mean Serum Anti-F Protein Ig Titers (Log.sub.10) Antigen Adjuvant (.mu.g) IgG IgG1 IgG2a IgA F protein NONE 2.6 .+-. 1.5 2.3 .+-. 1.3 2.0 .+-. 0.8 <1.7 F protein CT-CRM.sub.T48TH(1) 5.7 .+-. 0.1 5.4 .+-. 0.2 4.5 .+-. 0.3 4.0 .+-. 0.3 F protein CT-CRM.sub.T48TH(0.1) 4.3 .+-. 1.0 4.4 .+-. 1.0 3.5 .+-. 0.5 2.7 .+-. 0.9 F protein CT-CRM.sub.G34GCP(1) 5.8 .+-. 0.3 5.3 .+-. 0.2 4.9 .+-. 0.4 4.2 .+-. 0.2 F protein CT-CRM.sub.G34GCP(0.1) 5.4 .+-. 0.2 5.0 .+-. 0.3 4.1 .+-. 0.3 4.1 .+-. 0.2 F protein CT-CRM.sub.Y30WAH(1) 5.9 .+-. 0.4 5.1 .+-. 0.2 4.5 .+-. 0.3 3.9 .+-. 0.3 F protein CT-CRM.sub.Y30WAH(0.1) 4.7 .+-. 0.5 4.9 .+-. 0.4 3.6 .+-. 0.5 3.1 .+-. 0.5 F protein CT-CRM.sub.R25W(1) 6.1 .+-. 0.3 5.7 .+-. 0.3 4.5 .+-. 0.2 4.2 .+-. 0.2 F protein CT-CRM.sub.R25W(0.1) 5.5 .+-. 0.4 5.3 .+-. 0.4 4.2 .+-. 0.3 4.0 .+-. 0.1 F protein CT-CRM.sub.R25G(1) 5.4 .+-. 0.4 4.9 .+-. 0.6 4.0 .+-. 0.4 3.9 .+-. 0.2 F protein CT-CRM.sub.R25G(0.1) 3.8 .+-. 0.9 3.7 .+-. 0.8 3.0 .+-. 0.4 2.6 .+-. 0.8 F protein CT-CRM.sub.E29H(1) 5.9 .+-. 0.4 5.4 .+-. 0.4 4.8 .+-. 0.3 4.3 .+-. 0.1 F protein CT-CRM.sub.E29H(0.1) 5.9 .+-. 0.4 5.3 .+-. 0.2 4.5 .+-. 0.3 4.4 .+-. 0.3 F protein CT(1) 5.6 .+-. 1.2 5.2 .+-. 1.1 4.5 .+-. 1.1 4.3 .+-. 0.8 F protein CT(0.1) 5.0 .+-. 0.3 5.2 .+-. 0.3 4.5 .+-. 0.3 4.2 .+-. 0.2

TABLE-US-00021 TABLE 20 The Humoral Immune Response to BALB/c Mice after Intranasal Immunization with F Protein and Genetically Detoxified Mutants of CT Anti-F Protein Antibody Titers Anti- Adjuvant BAW NW VW gen (.mu.g) IgG IgA IgG IgA IgG IgA F protein NONE <25 <25 64 <25 <25 <25 F protein CT-CRM.sub.T48TH(1) 227 33 146 2,560 112 2,065 F protein CT-CRM.sub.T48TH(0.1) 59 27 <25 384 <25 344 F protein CT-CRM.sub.G34GCP(1) 964 458 60 708 125 562 F protein CT-CRM.sub.G34GCP(0.1) 181 <25 117 352 57 755 F protein CT-CRM.sub.Y30WAH(1) 312 <25 <25 177 52 1,332 F protein CT-CRM.sub.Y30WAH0.1) 111 24 63 210 <25 139 F protein CT-CRM.sub.R25W(1) 200 33 34 378 35 665 F protein CT-CRM.sub.R25W(0.1) 137 32 61 557 55 633 F protein CT-CRM.sub.R25G(1) 230 42 22 283 <25 307 F protein CT-CRM.sub.R25G(0.1) 44 <25 <25 39 <25 125 F protein CT-CRM.sub.E29H(1) 277 59 846 932 18 832 F protein CT-CRM.sub.E29H(0.1) 142 60 245 239 <25 496 F protein CT(1) 398 114 68 504 <25 981 F protein CT(0.1) 158 <25 <25 360 189 903

When the CT-CRM mutants of this invention were used as mucosal adjuvants at the 1.0 .mu.g dose, results similar to the use of mutant CT-CRM.sub.E29H or wild-type CT were obtained (Table 19). Noteworthy differences from the anti-F protein IgG or IgA titers elicited following immunization with F protein admixed with CT-CRM.sub.E29H or wild-type CT were not observed. However, at the 0.1 .mu.g dose, CT-CRM.sub.T48TH, CT-CRM.sub.Y30WAH and CT-CRM.sub.R25G appeared less able to augment serum anti-F protein IgA titers. The titers in the mucosal wash fluids of rice immunized with F protein formulated with the mutants of this invention appeared comparable to those induced by F protein admixed with CT-CRM.sub.E29H or wild-type CT (Table 20).

Thus, all CT-CRM mutants of this invention had adjuvant activity for F protein.

All publications and references cited in this specification are incorporated herein by reference. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

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6Vibrio cholerae l Lys Ile Ile Phe Val Phe Phe Ile Phe Leu Ser Ser Phe Serla Asn Asp Asp Lys Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp 2Glu Ile Lys Gln Ser Gly Gly Leu Met Pro Arg Gly Gln Ser Glu Tyr 35 4 Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg 5Gly Thr Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr65 7Ser Ile Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser 85 9 His Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Asn Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Glu Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Tyr Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg Gly Tyr Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala Asp Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Glu Glu Pro Trp Ile His His Ala Pro Pro Gly Cys Gly Asn Ala 2rg Ser Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu 222l Lys Phe Leu Asp Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile225 234r Gly Tyr Gln Ser Asp Ile Asp Thr His Asn Arg Ile Lys Asp 245 25u Leu Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu 267r Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys 275 28a Glu Ser His Asn Thr Gln Ile Tyr Thr Leu Asn Asp Lys Ile Phe 29yr Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr33he Lys Asn Gly Ala Ile Phe Gln Val Glu Val Pro Ser Ser Gln His 325 33e Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg 345a Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn 355 36n Lys Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn 378TVibrio cholerae 2Asn Asp Asp Lys Leu Tyr Arg Ala Asp Ser Arg Pro Pro Asp Glu Ileln Ser Gly Gly Leu Met Pro Arg Gly Gln Ser Glu Tyr Phe Asp 2Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr 35 4 Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr Ser Ile 5Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser Gly His65 7Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe Asn 85 9 Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Gln Glu Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg Gly Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala Ala Asp Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Arg Glu Pro Trp Ile His His Ala Pro Pro Gly Cys Gly Asn Ala Pro Arg Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu Gly Val 2he Leu Asp Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile Phe Ser 222r Gln Ser Asp Ile Asp Thr His Asn Arg Ile Lys Asp Glu Leu225 234artificial sequencebeta-amyloid peptide 3Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lysal Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile 2Gly Leu Met Val Gly Gly Val Val Ile Ala 35 4artificial sequencealpha-beta-amyloid peptide 4Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lysal Phe Phe Ala Glu Asp Val Gly Ser Asn Lys 2DNAArtificial sequenceprimer sequence 5ttttttgggc tagcatggag gaaaagatga gc 32626DNAArtificial sequenceprimer sequence 6cgaggtcgaa gcttgcatgt ttgggc 26

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